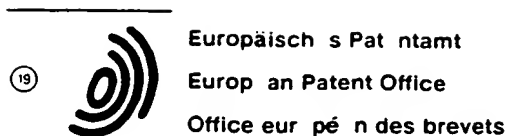


B7



Publication number: **0 318 554 B1**

**EUROPEAN PATENT SPECIFICATION**

(43) Date of publication of patent specification: 05.04.95 (51) Int. Cl. 6: **C07K 14/00, C12P 21/00, C12N 15/00, C07H 15/12**

(21) Application number: **88905298.1**

(22) Date of filing: **19.05.88**

(86) International application number:  
**PCT/US88/01737**

(87) International publication number:  
**WO 88/09344 (01.12.88 88/26)**

Divisional application 94201816.9 filed on  
19/05/88.

**(54) TARGETED MULTIFUNCTIONAL PROTEINS.**

(30) Priority: **21.05.87 US 52800**

(43) Date of publication of application:  
**07.06.89 Bulletin 89/23**

(45) Publication of the grant of the patent:  
**05.04.95 Bulletin 95/14**

(94) Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

(56) References cited:  
**EP-A- 0 290 005 WO-A-86/00090**  
**WO-A-86/01533 WO-A-88/01649**  
**WO-A-88/06630 US-A- 4 642 334**  
**US-A- 4 704 692**

**BIOTECHNOLOGY**, vol. 4, no. 12, December  
1986, New York, NY (US); **A. KLAUSNER**, pp.  
1041, 1043&NUM;

(73) Proprietor: **CREATIVE BIOMOLECULES, INC.**  
**35 South Street**  
**Hopkinton**  
**Massachusetts 01478 (US)**

(72) Inventor: **HUSTON, James, S.**  
**41 Whittemore Road**  
**Newton, MA 02158 (US)**  
Inventor: **OPPERMANN, Hermann**  
**25 Summerhill Road**  
**Medway, MA 02053 (US)**

(74) Representative: **Holdcroft, James Gerald, Dr.**  
**et al**  
**Graham Watt & Co.,**  
**Riverhead**  
**Sevenoaks, Kent TN13 2BN (GB)**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

BIOCHEMISTRY, vol. 17, September 1978, Washington, DC (US); M.S. ROSEMBLATT et al., pp. 3877-3882&NUM;

BIOCHEMISTRY, vol. 19, August 1980, Washington, DC (US); P.H. EHRLICH et al., pp. 4091-4096&NUM;

PROCEEDINGS OF THE NATL. ACADEMY OF SCIENCES USA, vol. 81, November 1984, Washington, DC (US); MORRISON et al., pp. 6851-6855&NUM;

PROCEEDINGS OF THE NATL. ACADEMY OF SCIENCES USA, vol. 84, January 1987, Washington, DC (US); L.K. SUN et al., pp. 214-218&NUM;

PROCEEDINGS OF THE NATL. ACADEMY OF SCIENCES USA, vol. 82, May 1985, Washington, DC (US); S. OHNO et al., pp. 2945-2949&NUM;

CHEMICAL ABSTRACTS, vol. 107, no. 5, 03 August 1987, Columbus, OH (US); A.P. RICHARDSON et al., p. 330, no. 35697n&NUM;

CHEMICAL ABSTRACTS, vol. 105, no. 5, 04 August 1986, Columbus, OH (US); R. ARNON, p. 1, no. 34903y&NUM;

CHEMICAL ABSTRACTS, vol. 106, no. 13, 30 March 1987, Columbus, OH (US); H.J. THIESEN et al., p. 37, no. 95759g&NUM;

CHEMICAL ABSTRACTS, vol. 105, no. 11, 15 September 1986, Columbus, OH (US); M.S. NEUBERGER, p. 475, no. 95609d&NUM;

PROCEEDINGS OF THE NATL. ACADEMY OF SCIENCES USA, vol. 81, June 1984, Washington, DC (US); S. CABILLY et al., pp. 3273-3277&NUM;

## D description

### Background of the Invention

5 This invention relates to novel compositions of matter, hereinafter called targeted multifunctional proteins, useful, for example, in specific binding assays, affinity purification, biocatalysis, drug targeting, imaging, immunological treatment of various oncogenic and infectious diseases, and in other contexts. More specifically, this invention relates to biosynthetic proteins expressed from recombinant DNA as a single polypeptide chain comprising plural regions, one of which has a structure similar to an antibody binding site, and an affinity for a preselected antigenic determinant, and another of which has a separate function, and may be biologically active, designed to bind to ions, or designed to facilitate immobilization of the protein. This invention also relates to the binding proteins per se, and methods for their construction.

There are five classes of human antibodies. Each has the same basic structure (see Figure 1), or multiple thereof, consisting of two identical polypeptides called heavy (H) chains (molecular weight approximately 50,000 d) and two identical light (L) chains (molecular weight approximately 25,000 d). Each of the five antibody classes has a similar set of light chains and a distinct set of heavy chains. A light chain is composed of one variable and one constant domain, while a heavy chain is composed of one variable and three or more constant domains. The combined variable domains of a paired light and heavy chain are known as the Fv region, or simply "Fv". The Fv determines the specificity of the immunoglobulin, the constant regions have other functions.

Amino acid sequence data indicate that each variable domain comprises three hypervariable regions or loops, sometimes called complementarity determining regions or "CDRs" flanked by four relatively conserved framework regions or "FRs" (Kabat et al., Sequences of Proteins of Immunological Interest [U.S. Department of Health and Human Services, third edition, 1983, fourth edition, 1987]). The hypervariable regions have been assumed to be responsible for the binding specificity of individual antibodies and to account for the diversity of binding of antibodies as a protein class.

Monoclonal antibodies have been used both as diagnostic and therapeutic agents. They are routinely produced according to established procedures by hybridomas generated by fusion of mouse lymphoid cells with an appropriate mouse myeloma cell line.

30 The literature contains a host of references to the concept of targeting bioactive substances such as drugs, toxins, and enzymes to specific points in the body to destroy or locate malignant cells or to induce a localized drug or enzymatic effect. It has been proposed to achieve this effect by conjugating the bioactive substance to monoclonal antibodies (see, e.g., Vogel, Immunoconjugates, Antibody Conjugates in Radioimaging and Therapy of Cancer, 1987, N.Y., Oxford University Press; and Ghose et al. (1978) *J. Natl. Cancer Inst.* 61:657-676, ). However, non-human antibodies induce an immune response when injected into humans. Human monoclonal antibodies may alleviate this problem, but they are difficult to produce by cell fusion techniques since, among other problems, human hybridomas are notably unstable, and removal of immunized spleen cells from humans is not feasible.

Chimeric antibodies composed of human and non-human amino acid sequences potentially have improved therapeutic value as they presumably would elicit less circulating human antibody against the non-human immunoglobulin sequences. Accordingly, hybrid antibody molecules have been proposed which consist of amino acid sequences from different mammalian sources. The chimeric antibodies designed thus far comprise variable regions from one mammalian source, and constant regions from human or another mammalian source (Morrison et al. (1984) *Proc. Natl. Acad. Sci. U.S.A.*, 81:5851-6855; Neuberger et al. (1984) *Nature* 312:604-608; Sahagan et al. (1986) *J. Immunol.* 137:1066-1074; EP-A-0 125 023, Genentech; EP-A-0 171 496 Research Development Corporation of Japan; EP-A-0 173 494 Stanford; WO-A-8 601 533 Celltech Limited).

It has been reported that binding function is localized to the variable domains of the antibody molecule located at the amino terminal end of both the heavy and light chains. The variable regions remain noncovalently associated (as V<sub>H</sub>V<sub>L</sub> dimers, termed Fv regions) even after proteolytic cleavage from the native antibody molecule, and retain much of their antigen recognition and binding capabilities (see, for example, Inbar et al., *Proc. Natl. Acad. Sci. U.S.A.* (1972) 69:2659-2662; Hochman et al. (1973) *Biochem.* 12:1130-1135; and (1976) *Biochem.* 15:2706-2710; Sharon and Givol (1976) *Biochem.* 15:1591-1594, Rosenblatt and Haber (1978) *Biochem.* 17:3877-3882; Ehrlich et al. (1980) *Biochem.* 19:4091-40996). Methods of manufacturing two-chain Fv substantially free of constant region using recombinant DNA techniques are disclosed in U.S. 4,642,334 and corresponding published specification EP 088,994. Bio-antibody alone or linked to an anti-cancer agent.

Summary of the Invention

In one aspect the invention provides a single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques, said protein comprising:

- 5 a biosynthetic antibody binding site capable of binding to a preselected antigenic determinant and comprising an amino acid sequence homologous with the sequence of a variable region of an immunoglobulin molecule capable of binding said preselected antigenic determinant.
- a first biofunctional domain comprising a polypeptide selected from the group consisting of effector proteins having a conformation suitable for biological activity in mammals, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support, and
- 10 a first polypeptide linker disposed between said binding site and said first biofunctional domain, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and which defines a polypeptide which connects the C-terminal end of said binding site and the N-terminal end of said first biofunctional domain or the N-terminal end of said binding site and the C-terminal end of said first
- 15 biofunctional domain, whereupon said binding protein assumes a conformation suitable for binding and said first biofunctional domain assumes a conformation suitable for biological activity, sequestering an ion, or selectively binding a solid support.

The binding site may comprise at least two binding domains peptide bonded by a second polypeptide linker disposed between said domains, wherein said second polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and which defines a polypeptide of a length sufficient to span the distance between the C-terminal end of one of said binding domains and the N-terminal end of the other of said binding domains when said binding protein assumes a conformation suitable for binding when disposed in aqueous solution.

The amino acid sequence of each of the binding domains may comprise a set of CDRs (Complementarity determining regions) interposed between a set of FRs (Framework regions), each of which is respectively homologous with CDRs and FRs from a said variable region of an immunoglobulin molecule capable of binding said preselected antigenic determinant.

At least one of said binding domains may comprise a said set of CDRs homologous with the CDRs in a first immunoglobulin and a set of FRs homologous with the FRs in a second, distinct immunoglobulin.

30 In another aspect, the invention provides a single polypeptide chain comprising:

- a pair of polypeptide domains together defining a site for binding a preselected antigen, and being joined through the C-terminus of one to the N-terminus of the other by a polypeptide linker, wherein the amino acid sequence of each of said polypeptide domains mimics an immunoglobulin variable region, and at least one said domain comprises:
- 35 a set of CDR amino acid sequences together defining a recognition site for said preselected antigen, wherein said CDR sequences are non-human sequences,
- a set of FR amino acid sequences linked to said set of CDR sequences, wherein said FR amino acid sequences are homologous to sequences obtained from a human immunoglobulin, and
- said linked sets of CDR and FR amino acid sequences together defining a chimeric binding domain
- 40 which, when disposed in aqueous solution, assumes a tertiary structure suitable for immunological binding with said preselected antigen.

In still another aspect, the invention provides a single polypeptide chain comprising:

- a pair of polypeptide domains defining a site for binding a preselected antigen joined by a polypeptide linker spanning the distance between the C-terminal of one to the N-terminal of the other, wherein the amino acid sequence of at least one of said polypeptide domains comprises a recombinant variable region
- 45 comprising:
- a set of CDR amino acid sequences together defining a recognition site for said preselected antigen, wherein said CDR sequences are homologous to sequences obtained from a first immunoglobulin,
- a set of FR amino acid sequences linked to said set of CDR sequences, wherein said FR amino acid sequences are homologous to sequences obtained from a second immunoglobulin, and
- 50 said linked sets of CDR and FR amino acid sequences together defining a chimeric single chain variable region binding polypeptide which, when disposed in aqueous solution, assumes a tertiary structure suitable for immunological binding with said preselected antigen.

In preferred aspects, the FRs of the binding protein are homologous to at least a portion of the FRs from a human immunoglobulin, the linker spans at least about 40 angstroms; a polypeptide spacer is incorporated in the multifunctional protein between the binding site and the second polypeptide; and the binding protein has an affinity for the preselected antigenic determinant no less than two orders of magnitude less than the binding affinity of the immunoglobulin molecule used as a template for the CDR

regions of the binding protein. The preferred linkers and spacers are cysteine-free. The linker preferably comprises amino acids having unreactive side groups, e.g., alanin and glycine. Linkers and spacers can be made by combining plural consecutive copies of an amino acid sequence, e.g., (Gly<sub>4</sub> Ser)<sub>3</sub>. The invention also provides DNAs encoding these proteins and host cells harboring and capable of expressing these

5 DNAs.

As used herein, the phrase biosynthetic antibody binding site or BABS means synthetic proteins expressed from DNA derived by recombinant techniques. BABS comprise biosynthetically produced sequences of amino acids defining polypeptides designed to bind with a preselected antigenic material. The structure of these synthetic polypeptides is unlike that of naturally occurring antibodies, fragments thereof, e.g., Fv, or known synthetic polypeptides or "chimeric antibodies" in that the regions of the BABS responsible for specificity and affinity of binding, (analogous to native antibody variable regions) are linked by peptide bonds, expressed from a single DNA, and may themselves be chimeric, e.g., may comprise amino acid sequences homologous to portions of at least two different antibody molecules. The BABS embodying the invention are biosynthetic in the sense that they are synthesized in a cellular host made to express a synthetic DNA, that is, a recombinant DNA made by ligation of plural, chemically synthesized oligonucleotides, or by ligation of fragments of DNA derived from the genome of a hybridoma, mature B cell clone, or a cDNA library derived from such natural sources. The proteins of the invention are properly characterized as "binding sites" in that these synthetic molecules are designed to have specific affinity for a preselected antigenic determinant. The polypeptides of the invention comprise structures patterned after

20 regions of native antibodies known to be responsible for antigen recognition.

Accordingly, it is an object of the invention to provide novel multifunctional proteins comprising one or more effector proteins and one or more biosynthetic antibody binding sites, and to provide DNA sequences which encode the proteins. Another object is to provide a generalized method for producing biosynthetic antibody binding site polypeptides of any desired specificity.

25

#### Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings.

Figure 1A is a schematic representation of an intact IgG antibody molecule containing two light chains, each consisting of one variable and one constant domain, and two heavy chains, each consisting of one variable and three constant domains. Figure 1B is a schematic drawing of the structure of Fv proteins (and DNA encoding them) illustrating V<sub>H</sub> and V<sub>L</sub> domains, each of which comprises four framework (FR) regions and three complementarity determining (CDR) regions. Boundaries of CDRs are indicated, by way of example, for monoclonal 26-10, a well known and characterized murine monoclonal specific for digoxin.

35 and three complementarity determining (CDR) regions. Boundaries of CDRs are indicated, by way of example, for monoclonal 26-10, a well known and characterized murine monoclonal specific for digoxin.

Figure 2A-2E are schematic representations of some of the classes of reagents constructed in accordance with the invention, each of which comprises a biosynthetic antibody binding site.

Figure 3 discloses five amino acid sequences (heavy chains) in single letter code lined up vertically to facilitate understanding of the invention. Sequence 1 is the known native sequence of V<sub>H</sub> from murine monoclonal glp-4 (anti-lysozyme). Sequence 2 is the known native sequence of V<sub>H</sub> from murine monoclonal 26-10 (anti-digoxin). Sequence 3 is a BABS comprising the FRs from 26-10 V<sub>H</sub> and the CDRs from glp-4 V<sub>H</sub>. The CDRs are identified in lower case letters; restriction sites in the DNA used to produce chimeric sequence 3 are also identified. Sequence 4 is the known native sequence of V<sub>H</sub> from human myeloma antibody NEWM. Sequence 5 is a BABS comprising the FRs from NEWM V<sub>H</sub> and the CDRs from glp-4 V<sub>H</sub>, i.e., illustrates a "humanized" binding site having a human framework but an affinity for lysozyme similar to murine glp-4.

Figures 4A-4F are the synthetic nucleic acid sequences and encoded amino acid sequences of (4A) the heavy chain variable domain of murine anti-digoxin monoclonal 26-10; (4B) the light chain variable domain of murine anti-digoxin monoclonal 26-10; (4C) a heavy chain variable domain of a BABS comprising CDRs of glp-4 and FRs of 26-10; (4D) a light chain variable region of the same BABS; (4E) a heavy chain variable region of a BABS comprising CDRs of glp-4 and FRs of NEWM; and (4F) a light chain variable region comprising CDRs of glp-4 and FRs of NEWM. Delineated are FRs, CDRs, and restriction sites for endonuclease digestion, most of which were introduced during design of the DNA.

55 Figure 5 is the nucleic acid and encoded amino acid sequence of a host DNA (V<sub>H</sub>) designed to facilitate insertion of CDRs of choice. The DNA was designed to have unique 6-base sites directly flanking the CDRs so that relatively small oligonucleotides defining portions of CDRs can be readily inserted, and to have other sites to facilitate manipulation of the DNA to optimize binding properties in a given construct. The

framework regions of the molecule correspond to murine FRs (Figure 4A).

Figures 6A and 6B are multifunctional proteins (and DNA encoding them) comprising a single chain BABS with the specificity of murine monoclonal 26-10, linked through a spacer to the FB fragment of protein A, here fused as a leader, and constituting a binding site for Fc. The spacer comprises the 11 C-terminal amino acids of the FB followed by Asp-Pro (a dilute acid cleavage site). The single chain BABS comprises sequences mimicking the  $V_H$  and  $V_L$  (6A) and the  $V_L$  and  $V_H$  (6B) of murine monoclonal 26-10. The  $V_L$  in construct 6A is altered at residue 4 where valine replaces methionine present in the parent 26-10 sequence. These constructs contain binding sites for both Fc and digoxin. Their structure may be summarized as:

(6A) FB-Asp-Pro- $V_H$ -(Gly-Ser)<sub>3</sub>- $V_L$ .

and

(6B) FB-Asp-Pro- $V_L$ -(Gly-Ser)<sub>3</sub>- $V_H$ .

where (Gly-Ser)<sub>3</sub> is a polypeptide linker.

In Figures 4A-4E and 6A and 6B, the amino acid sequence of the expression products start after the GAATTC sequences, which codes for an EcoRI splice site, translated as Glu-Phe on the drawings.

Figure 7A is a graph of percent of maximum counts bound of radiolabeled digoxin versus concentration of binding protein adsorbed to the plate comparing the binding of native 26-10 (curve 1) and the construct of Figure 6A and Figure 2B renatured using two different procedures (curves 2 and 3). Figure 7B is a graph demonstrating the bifunctionality of the FB-(26-10) BABS adhered to microtiter plates through the specific binding of the binding site to the digoxin-BSA coat on the plate. Figure 7B shows the percent inhibition of <sup>125</sup>I-rabbit-IgG binding to the FB domain of the FB BABS by the addition of IgG, protein A, FB, murine IgG2a, and murine IgG1.

Figure 8 is a schematic representation of a model assembled DNA sequence encoding a multifunctional biosynthetic protein comprising a leader peptide (used to aid expression and thereafter cleaved), a binding site, a spacer, and an effector molecule attached as a trailer sequence.

Figure 9A-9E are exemplary synthetic nucleic acid sequences and corresponding encoded amino acid sequences of binding sites of different specificities: (A) FRs from NEWM and CDRs from 26-10 having the digoxin specificity of murine monoclonal 26-10; (B) FRs from 26-10, and CDRs from G-loop-4 (glp-4) having lysozyme specificity; (C) FRs and CDRs from MOPC-315 having dinitrophenol (DNF) specificity; (D) FRs and CDRs from an anti-CEA monoclonal antibody; (E) FRs in both  $V_H$  and  $V_L$  and CDR<sub>1</sub> and CDR<sub>3</sub> in  $V_H$ , and CDR<sub>1</sub>, CDR<sub>2</sub>, and CDR<sub>3</sub> in  $V_L$  from an anti-CEA monoclonal antibody; CDR<sub>2</sub> in  $V_H$  is a CDR<sub>2</sub> consensus sequence found in most immunoglobulin  $V_H$  regions.

Figure 10A is a schematic representation of the DNA and amino acid sequence of a leader peptide (MLE) protein with corresponding DNA sequence and some major restriction sites. Figure 10B shows the design of an expression plasmid used to express MLE-BABS (26-10). During construction of the gene, fusion partners were joined at the EcoRI site that is shown as part of the leader sequence. The pBR322 plasmid, opened at the unique SspI and PstI sites, was combined in a 3-part ligation with an SspI to EcoRI fragment bearing the *trp* promoter and MLE leader and with an EcoRI to PstI fragment carrying the BABS gene. The resulting expression vector confers tetracycline resistance on positive transformants.

Figure 11 is an SDS-polyacrylamide gel (15%) of the (26-10) BABS at progressive stages of purification. Lane 0 shows low molecular weight standards; lane 1 is the MLE-BABS fusion protein; lane 2 is an acid digest of this material; lane 3 is the pooled DE-52 chromatographed protein; lanes 4 and 5 are the same ouabain-Sepharose® pool of single chain BABS except that lane 4 protein is reduced and lane 5 protein is unreduced.

Figure 12 shows inhibition curves for 26-10 BABS and 26-10 Fab species, and indicates the relative affinities of the antibody fragment for the indicated cardiac glycosides.

Figures 13A and 13B are plots of digoxin binding curves. (A) shows 26-10 BABS binding isotherm and Sips plot (inset), and (B) shows 26-10 Fab binding isotherm and Sips plot (inset).

Figure 14 is a nucleic acid sequence and corresponding amino acid sequence of a modified FB dimer leader sequence and various restriction sites.

Figure 15A-15H are nucleic acid sequences and corresponding amino acid sequences of biosynthetic multifunctional proteins including a single chain BABS and various biologically active protein trailers linked via a spacer sequence. Also indicated are various endonuclease digestion sites. The trailing sequences are (A) epidermal growth factor (EGF); (B) streptavidin; (C) tumor necrosis factor (TNF); (D) calmodulin; (E) platelet derived growth factor-beta (PDGF-beta); (F) ricin; and (G) interleukin-2, and (H) an FB-FB dimer.

Description

The invention will first be described in its broadest overall aspects with a more detailed description following.

5 A class of novel biosynthetic, bi or multifunctional proteins has now been designed and engineered which comprise biosynthetic antibody binding sites, that is, "BABS" or biosynthetic polypeptides defining structure capable of selective antigen recognition and preferential antigen binding, and one or more peptide-bonded additional protein or polypeptide regions designed to have a preselected property. Examples of the  
 10 second region include amino acid sequences designed to sequester ions, which makes the protein suitable for use as an imaging agent, and sequences designed to facilitate immobilization of the protein for use in affinity chromatography and solid phase immunoassay. Another example of the second region is a bioactive effector molecule, that is, a protein having a conformation suitable for biological activity, such as an enzyme, toxin, receptor, binding site, growth factor, cell differentiation factor, lymphokine, cytokine, hormone, or anti-metabolite. This invention features synthetic, multifunctional proteins comprising these  
 15 regions peptide bonded to one or more biosynthetic antibody binding sites, synthetic, single chain proteins designed to bind preselected antigenic determinants with high affinity and specificity, constructs containing multiple binding sites linked together to provide multipoint antigen binding and high net affinity and specificity, DNA encoding these proteins prepared by recombinant techniques, host cells harboring these DNAs, and methods for the production of these proteins and DNAs.

20 The invention requires recombinant production of single chain binding sites having affinity and specificity for a predetermined antigenic determinant. This technology has been developed and is disclosed herein. In view of this disclosure, persons skilled in recombinant DNA technology, protein design, and protein chemistry can produce such sites which, when disposed in solution, have high binding constants (at least  $10^6$ , preferably  $10^8$   $M^{-1}$ .) and excellent specificity.

25 The design of the BABS is based on the observation that three subregions of the variable domain of each of the heavy and light chains of native immunoglobulin molecules collectively are responsible for antigen recognition and binding. Each of these subregions, called herein "complementarity determining regions" or CDRs, consists of one of the hypervariable regions or loops and of selected amino acids or amino acid sequences disposed in the framework regions or FRs which flank that particular hypervariable  
 30 region. It has now been discovered that FRs from diverse species are effective to maintain CDRs from diverse other species in proper conformation so as to achieve true immunochemical binding properties in a biosynthetic protein. It has also been discovered that biosynthetic domains mimicking the structure of the two chains of an immunoglobulin binding site may be connected by a polypeptide linker while closely approaching, retaining, and often improving their collective binding properties.

35 The binding site region of the multifunctional proteins comprises at least one, and preferably two domains, each of which has an amino acid sequence homologous to portions of the CDRs of the variable domain of an immunoglobulin light or heavy chain, and other sequence homologous to the FRs of the variable domain of the same, or a second, different immunoglobulin light or heavy chain. The two domain binding site construct also includes a polypeptide linking the domains. Polypeptides so constructed bind a  
 40 specific preselected antigen determined by the CDRs held in proper conformation by the FRs and the linker. Preferred structures have human FRs, i.e., mimic the amino acid sequence of at least a portion of the framework regions of a human immunoglobulin, and have linked domains which together comprise structure mimicking a  $V_H$ - $V_L$  or  $V_L$ - $V_H$  immunoglobulin two-chain binding site. CDR regions of a mammalian immunoglobulin, such as those of mouse, rat, or human origin are preferred. In one preferred embodiment,  
 45 the biosynthetic antibody binding site comprises FRs homologous with a portion of the FRs of a human immunoglobulin and CDRs homologous with CDRs from a mouse or rat immunoglobulin. This type of chimeric polypeptide displays the antigen binding specificity of the mouse or rat immunoglobulin, while its human framework minimizes human immune reactions. In addition, the chimeric polypeptide may comprise other amino acid sequences. It may comprise, for example, a sequence homologous to a portion of the  
 50 constant domain of an immunoglobulin, but preferably is free of constant regions (other than FRs).

The binding site region(s) of the chimeric proteins are thus single chain composite polypeptides comprising a structure which in solution behaves like an antibody binding site. The two domain, single chain composite polypeptide has a structure patterned after tandem  $V_H$  and  $V_L$  domains, but with the carboxyl terminal of one attached through a linking amino acid sequence to the amino terminal of the other. The  
 55 linking amino acid sequence may or may not itself be antigenic or biologically active. It preferably spans a distance of at least about 40A, i.e., comprises at least about 14 amino acids, and comprises residues which together present a hydrophilic, relatively unstructured region. Linking amino acid sequences having little or no secondary structure work well. Optionally, one or a pair of unique amino acids or amino acid sequences

recognizable by a site specific cleavage agent may be included in the linker. This permits the  $V_H$  and  $V_L$ -like domains to be separated after expression, or the linker to be excised after refolding of the binding site.

Either the amino or carboxyl terminal ends (or both ends) of these chimeric, single chain binding sites are attached to an amino acid sequence which itself is bioactive or has some other function to produce a bifunctional or multifunctional protein. For example, the synthetic binding site may include a leader and/or trailer sequence defining a polypeptide having enzymatic activity, independent affinity for an antigen different from the antigen to which the binding site is directed, or having other functions such as to provide a convenient site of attachment for a radioactive ion, or to provide a residue designed to link chemically to a solid-support. This fused, independently functional section of protein should be distinguished from fused leaders used simply to enhance expression in prokaryotic host cells or yeasts. The multifunctional proteins also should be distinguished from the "conjugates" disclosed in the prior art comprising antibodies which, after expression, are linked chemically to a second moiety.

Often, a series of amino acids designed as a "spacer" is interposed between the active regions of the multifunctional protein. Use of such a spacer can promote independent refolding of the regions of the protein. The spacer also may include a specific sequence of amino acids recognized by an endopeptidase, for example, endogenous to a target cell (e.g., one having a surface protein recognized by the binding site) so that the bioactive effector protein is cleaved and released at the target. The second functional protein preferably is present as a trailer sequence, as trailers exhibit less of a tendency to interfere with the binding behavior of the BABS.

The therapeutic use of such "self-targeted" bioactive proteins offers a number of advantages over conjugates of immunoglobulin fragments or complete antibody molecules: they are stable, less immunogenic and have a lower molecular weight; they can penetrate body tissues more rapidly for purposes of imaging or drug delivery because of their smaller size; and they can facilitate accelerated clearance of targeted isotopes or drugs. Furthermore, because design of such structures at the DNA level as disclosed herein permits ready selection of bioproperties and specificities, an essentially limitless combination of binding sites and bioactive proteins is possible, each of which can be refined as disclosed herein to optimize independent activity at each region of the synthetic protein. The synthetic proteins can be expressed in prokaryotes such as *E. coli*, and thus are less costly to produce than immunoglobulins or fragments thereof which require expression in cultured animal cell lines.

The invention thus provides a family of recombinant proteins expressed from a single piece of DNA, all of which have the capacity to bind specifically with a predetermined antigenic determinant. The preferred species of the proteins comprise a second domain which functions independently of the binding region. In this aspect the invention provides an array of "self-targeted" proteins which have a bioactive function and which deliver that function to a locus determined by the binding site's specificity. It also provides biosynthetic binding proteins having attached polypeptides suitable for attachment to immobilization matrices which may be used in affinity chromatography and solid phase immunoassay applications, or suitable for attachment to ions, e.g., radioactive ions, which may be used for in vivo imaging.

The successful design and manufacture of the proteins of the invention depends on the ability to produce biosynthetic binding sites, and most preferably, sites comprising two domains mimicking the variable domains of immunoglobulin connected by a linker.

As is now well known, Fv, the minimum antibody fragment which contains a complete antigen recognition and binding site, consists of a dimer of one heavy and one light chain variable domain in noncovalent association (Figure 1A). It is in this configuration that the three complementarity determining regions of each variable domain interact to define an antigen binding site on the surface of the  $V_H$ - $V_L$  dimer. Collectively, the six complementarity determining regions (see Figure 1B) confer antigen binding specificity to the antibody. FRs flanking the CDRs have a tertiary structure which is essentially conserved in native immunoglobulins of species as diverse as human and mouse. These FRs serve to hold the CDRs in their appropriate orientation. The constant domains are not required for binding function, but may aid in stabilizing  $V_H$ - $V_L$  interaction. Even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than an entire binding site (Painter et al. (1972) *Biochem.* 11:1327-1337).

This knowledge of the structure of immunoglobulin proteins has now been exploited to develop multifunctional fusion proteins comprising biosynthetic antibody binding sites and one or more other domains.

The structure of these biosynthetic proteins in the region which impart the binding properties to the protein is analogous to the Fv region of a natural antibody. It comprises at least one, and preferably two domains consisting of amino acids defining  $V_H$  and  $V_L$ -like polypeptide segments connected by a linker which together form the tertiary molecular structure responsible for affinity and specificity. Each domain



comprises a set of amino acid sequences analogous to immunoglobulin CDRs held in appropriate conformation by a set of sequences analogous to the framework regions (FRs) of an Fv fragment of a natural antibody.

The term CDR, as used herein, refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site, or a synthetic polypeptide which mimics this function. CDRs typically are not wholly homologous to hypervariable regions of natural Fvs, but rather also may include specific amino acids or amino acid sequences which flank the hypervariable region and have heretofore been considered framework not directly determinative of complementarity. The term FR, as used herein, refers to amino acid sequences flanking or interposed between CDRs.

The CDR and FR polypeptide segments are designed based on sequence analysis of the Fv region of preexisting antibodies or of the DNA encoding them. In one embodiment, the amino acid sequences constituting the FR regions of the BABS are analogous to the FR sequences of a first preexisting antibody, for example, a human IgG. The amino acid sequences constituting the CDR regions are analogous to the sequences from a second, different preexisting antibody, for example, the CDRs of a murine IgG. Alternatively, the CDRs and FRs from a single preexisting antibody from, e.g., an unstable or hard to culture hybridoma, may be copied in their entirety.

Practice of the invention enables the design and biosynthesis of various reagents, all of which are characterized by a region having affinity for a preselected antigenic determinant. The binding site and other regions of the biosynthetic protein are designed with the particular planned utility of the protein in mind. Thus, if the reagent is designed for intravascular use in mammals, the FR regions may comprise amino acids similar or identical to at least a portion of the framework region amino acids of antibodies native to that mammalian species. On the other hand, the amino acids comprising the CDRs may be analogous to a portion of the amino acids from the hypervariable region, (and certain flanking amino acids) of an antibody having a known affinity and specificity, e.g., a murine or rat monoclonal antibody.

Other sections of native immunoglobulin protein structure, e.g.,  $C_H$  and  $C_L$ , need not be present and normally are intentionally omitted from the biosynthetic proteins. However, the proteins of the invention normally comprise additional polypeptide or protein regions defining a bioactive region, e.g., a toxin or enzyme, or a site onto which a toxin or a remotely detectable substance can be attached.

The invention thus can provide intact biosynthetic antibody binding sites analogous to  $V_H$ - $V_L$  dimers, either non-covalently associated, disulfide bonded, or preferably linked by a polypeptide sequence to form a composite  $V_H$ - $V_L$  or  $V_L$ - $V_H$  polypeptide which may be essentially free of antibody constant region. The invention also provides proteins analogous to an independent  $V_H$  or  $V_L$  domain, or dimers thereof. Any of these proteins may be provided in a form linked to, for example, amino acids analogous or homologous to a bioactive molecule such as a hormone or toxin.

Connecting the independently functional regions of the protein is a spacer comprising a short amino acid sequence whose function is to separate the functional regions so that they can independently assume their active tertiary conformation. The spacer can consist of an amino acid sequence present on the end of a functional protein which sequence is not itself required for its function, and/or specific sequences engineered into the protein at the DNA level.

The spacer generally may comprise between 5 and 25 residues. Its optimal length may be determined using constructs of different spacer lengths varying, for example, by units of 5 amino acids. The specific amino acids in the spacer can vary. Cysteines should be avoided. Hydrophilic amino acids are preferred. The spacer sequence may mimic the sequence of a hinge region of an immunoglobulin. It may also be designed to assume a structure, such as a helical structure. Proteolytic cleavage sites may be designed into the spacer separating the variable region-like sequences from other pendant sequences so as to facilitate cleavage of intact BABS, free of other protein, or so as to release the bioactive protein in vivo.

Figures 2A-2E illustrate five examples of protein structures embodying the invention that can be produced by following the teaching disclosed herein. All are characterized by a biosynthetic polypeptide defining a binding site 3, comprising amino acid sequences comprising CDRs and FRs, often derived from different immunoglobulins, or sequences homologous to a portion of CDRs and FRs from different immunoglobulins. Figure 2A depicts a single chain construct comprising a polypeptide domain 10 having an amino acid sequence analogous to the variable region of an immunoglobulin heavy chain, bound through its carboxyl end to a polypeptide linker 12, which in turn is bound to a polypeptide domain 14 having an amino acid sequence analogous to the variable region of an immunoglobulin light chain. Of course, the light and heavy chain domains may be in reverse order. Alternatively, the binding site may comprise two substantially homologous amino acid sequences which are both analogous to the variable region of an immunoglobulin heavy or light chain.

The linker 12 should be long enough (e.g., about 15 amino acids or about 40 Å to permit the chains 10 and 14 to assume their proper conformation. The linker 12 may comprise an amino acid sequence homologous to a sequence identified as "self" by the species into which it will be introduced, if drug use is intended. For example, the linker may comprise an amino acid sequence patterned after a hinge region of an immunoglobulin. The linker preferably comprises hydrophilic amino acid sequences. It may also comprise a bioactive polypeptide such as a cell toxin which is to be targeted by the binding site, or a segment easily labelled by a radioactive reagent which is to be delivered, e.g., to the site of a tumor comprising an epitope recognized by the binding site. The linker may also include one or two built-in cleavage sites, i.e., an amino acid or amino acid sequence susceptible to attack by a site-specific cleavage agent as described below. This strategy permits the  $V_H$  and  $V_L$ -like domains to be separated after expression, or the linker to be excised after folding while retaining the binding site structure in non-covalent association. The amino acids of the linker preferably are selected from among those having relatively small, unreactive side chains. Alanine, serine, and glycine are preferred.

Generally, the design of the linker involves considerations similar to the design of the spacer, excepting that binding properties of the linked domains are seriously degraded if the linker sequence is shorter than about 20Å in length, i.e., comprises less than about 10 residues. Linkers longer than the approximate 40Å distance between the N terminal of a native variable region and the C-terminal of its sister chain may be used, but also potentially can diminish the BABS binding properties. Linkers comprising between 12 and 18 residues are preferred. The preferred length in specific constructs may be determined by varying linker length first by units of 5 residues, and second by units of 1-4 residues after determining the best multiple of the pentameric starting units.

Additional proteins or polypeptides may be attached to either or both the amino or carboxyl termini of the binding site to produce multifunctional proteins of the type illustrated in Figures 2B-2E. As an example, in Figure 2B, a helically coiled polypeptide structure 16 comprises a protein A fragment (FB) linked to the amino terminal end of a  $V_H$ -like domain 10 via a spacer 18. Figure 2C illustrates a bifunctional protein having an effector polypeptide 20 linked via spacer 22 to the carboxyl terminus of polypeptide 14 of binding protein segment 2. This effector polypeptide 20 may consist of, for example, a toxin, therapeutic drug, binding protein, enzyme or enzyme fragment, site of attachment for an imaging agent (e.g., to chelate a radioactive ion such as indium), or site of selective attachment to an immobilization matrix so that the BABS can be used in affinity chromatography or solid phase binding assay. This effector alternatively may be linked to the amino terminus of polypeptide 10, although trailers are preferred. Figure 2D depicts a trifunctional protein comprising a linked pair of BABS 2 having another distinct protein domain 20 attached to the N-terminus of the first binding protein segment. Use of multiple BABS in a single protein enables production of constructs having very high selective affinity for multi-epitopic sites such as cell surface proteins.

The independently functional domains are attached by a spacer 18 (Figs 2B and 2D) covalently linking the C terminus of the protein 16 or 20 to the N-terminus of the first domain 10 of the binding protein segment 2, or by a spacer 22 linking the C-terminus of the second binding domain 14 to the N-terminus of another protein (Figs. 2C and 2D). The spacer may be an amino acid sequence analogous to linker sequence 12, or it may take other forms. As noted above, the spacer's primary function is to separate the active protein regions to promote their independent bioactivity and permit each region to assume its bioactive conformation independent of interference from its neighboring structure.

Figure 2E depicts another type of reagent, comprising a BABS having only one set of three CDRs, e.g., analogous to a heavy chain variable region, which retains a measure of affinity for the antigen. Attached to the carboxyl end of the polypeptide 10 or 14 comprising the FR and CDR sequences constituting the binding site 3 through spacer 22 is effector polypeptide 20 as described above.

As is evidenced from the foregoing, the invention provides a large family of reagents comprising proteins, at least a portion of which defines a binding site patterned after the variable region of an immunoglobulin. It will be apparent that the nature of any protein fragments linked to the BABS, and used for reagents embodying the invention, are essentially unlimited, the essence of the invention being the provision, either alone or linked to other proteins, of binding sites having specificities to any antigen desired.

The clinical administration of multifunctional proteins comprising a BABS, or a BABS alone, affords a number of advantages over the use of intact natural or chimeric antibody molecules, fragments thereof, and conjugates comprising such antibodies linked chemically to a second bioactive moiety. The multifunctional proteins described herein offer fewer cleavage sites to circulating proteolytic enzymes, their functional domains are connected by peptide bonds to polypeptide linker or spacer sequences, and thus the proteins have improved stability. Because of their smaller size and efficient design, the multifunctional proteins described herein reach their target tissue more rapidly, and are cleared more quickly from the body. They

also have reduced immunogenicity. In addition, their design facilitates coupling to other moieties in drug targeting and imaging application. Such coupling may be conducted chemically after expression of the BABS to a site of attachment for the coupling product engineered into the protein at the DNA level. Active effector proteins having toxic, enzymatic, binding, modulating, cell differentiating, hormonal, or other bioactivity are expressed from a single DNA as a leader and or trailer sequence, peptide bonded to the BABS.

#### Design and Manufacture

The proteins of the invention are designed at the DNA level. The chimeric or synthetic DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured if necessary. A preferred general structure of the DNA encoding the proteins is set forth in Figure 8. As illustrated, it encodes an optimal leader sequence used to promote expression in procaryotes having a built-in cleavage site recognizable by a site specific cleavage agent, for example, an endopeptidase, used to remove the leader after expression. This is followed by DNA encoding a  $V_L$ -like domain, comprising CDRs and FRs, a linker, a  $V_H$ -like domain, again comprising CDRs and FRs, a spacer, and an effector protein. After expression, folding, and cleavage of the leader, a bifunctional protein is produced having a binding region whose specificity is determined by the CDRs, and a peptide-linked independently functional effector region.

The ability to design the BABS of the invention depends on the ability to determine the sequence of the amino acids in the variable region of monoclonal antibodies of interest, or the DNA encoding them. Hybridoma technology enables production of cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma. The 5' end portion of the mRNA can be used to prepare cDNA for subsequent sequencing, or the amino acid sequence of the hypervariable and flanking framework regions can be determined by amino acid sequencing of the V region fragments of the H and L chains. Such sequence analysis is now conducted routinely. This knowledge, coupled with observations and deductions of the generalized structure of immunoglobulin Fvs, permits one to design synthetic genes encoding FR and CDR sequences which likely will bind the antigen. These synthetic genes are then prepared using known techniques, or using the technique disclosed below, inserted into a suitable host, and expressed, and the expressed protein is purified. Depending on the host cell, renaturation techniques may be required to attain proper conformation. The various proteins are then tested for binding ability, and one having appropriate affinity is selected for incorporation into a reagent of the type described above. If necessary, point substitutions seeking to optimize binding may be made in the DNA using conventional cassette mutagenesis or other protein engineering methodology such as is disclosed below.

Preparation of the proteins of the invention also is dependent on knowledge of the amino acid sequence (or corresponding DNA or RNA sequence) of bioactive proteins such as enzymes, toxins, growth factors, cell differentiation factors, receptors, anti-metabolites, hormones or various cytokines or lymphokines. Such sequences are reported in the literature and available through computerized data banks.

The DNA sequences of the binding site and the second protein domain are fused using conventional techniques, or assembled from synthesized oligonucleotides, and then expressed using equally conventional techniques.

The processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art, and therefore, not described in detail herein. Methods of identifying and isolating genes encoding antibodies of interest are well understood, and described in the patent and other literature. In general, the methods involve selecting genetic material coding for amino acids which define the proteins of interest, including the CDRs and FRs of interest, according to the genetic code.

Accordingly, the construction of DNAs encoding proteins as disclosed herein can be done using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and synthetic probes for isolating immunoglobulin or other bioactive protein genes. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known to those skilled in the art. Various types of vectors may be used such as plasmids and viruses including animal viruses and bacteriophages. The vectors may exploit various marker genes which impart to a successfully transfected cell a detectable phenotypic property that can be used to identify which of a family of clones has successfully incorporated the recombinant DNA of the

vector.

One method for obtaining DNA encoding the proteins disclosed herein is by assembly of synthetic oligonucleotides produced in a conventional, automated, polynucleotide synthesizer followed by ligation with appropriate ligases. For example, overlapping, complementary DNA fragments comprising 15 bases may be synthesized semi manually using phosphoramidite chemistry, with end segments left unphosphorylated to prevent polymerization during ligation. One end of the synthetic DNA is left with a "sticky end" corresponding to the site of action of a particular restriction endonuclease, and the other end is left with an end corresponding to the site of action of another restriction endonuclease. Alternatively, this approach can be fully automated. The DNA encoding the protein may be created by synthesizing longer single strand fragments (e.g., 50-100 nucleotides long) in, for example, a Biosearch oligonucleotide synthesizer, and then ligating the fragments.

A method of producing the BABS of the invention is to produce a synthetic DNA encoding a polypeptide comprising, e.g., human FRs, and intervening "dummy" CDRs, or amino acids having no function except to define suitably situated unique restriction sites. This synthetic DNA is then altered by DNA replacement, in which restriction and ligation is employed to insert synthetic oligonucleotides encoding CDRs defining a desired binding specificity in the proper location between the FRs. This approach facilitates empirical refinement of the binding properties of the BABS.

This technique is dependent upon the ability to cleave a DNA corresponding in structure to a variable domain gene at specific sites flanking nucleotide sequences encoding CDRs. These restriction sites in some cases may be found in the native gene. Alternatively, non-native restriction sites may be engineered into the nucleotide sequence resulting in a synthetic gene with a different sequence of nucleotides than the native gene, but encoding the same variable region amino acids because of the degeneracy of the genetic code. The fragments resulting from endonuclease digestion, and comprising FR-encoding sequences, are then ligated to non-native CDR-encoding sequences to produce a synthetic variable domain gene with altered antigen binding specificity. Additional nucleotide sequences encoding, for example, constant region amino acids or a bioactive molecule may then be linked to the gene sequences to produce a bifunctional protein.

The expression of these synthetic DNA's can be achieved in both prokaryotic and eucaryotic systems via transfection with an appropriate vector. In *E. coli* and other microbial hosts, the synthetic genes can be expressed as fusion protein which is subsequently cleaved. Expression in eucaryotes can be accomplished by the transfection of DNA sequences encoding CDR and FR region amino acids and the amino acids defining a second function into a myeloma or other type of cell line. By this strategy intact hybrid antibody molecules having hybrid Fv regions and various bioactive proteins including a biosynthetic binding site may be produced. For fusion protein expressed in bacteria, subsequent proteolytic cleavage of the isolated fusions can be performed to yield free BABS, which can be renatured to obtain an intact biosynthetic, hybrid antibody binding site.

Heretofore, it has not been possible to cleave the heavy and light chain region to separate the variable and constant regions of an immunoglobulin so as to produce intact Fv, except in specific cases not of commercial utility. However, one method of producing BABS in accordance with this invention is to redesign DNAs encoding the heavy and light chains of an immunoglobulin, optionally altering its specificity or humanizing its FRs, and incorporating a cleavage site and "hinge region" between the variable and constant regions of both the heavy and light chains. Such chimeric antibodies can be produced in transfectomas or the like and subsequently cleaved using a preselected endopeptidase.

The hinge region is a sequence of amino acids which serve to promote efficient cleavage by a preselected cleavage agent at a preselected, built-in cleavage site. It is designed to promote cleavage preferentially at the cleavage site when the polypeptide is treated with the cleavage agent in an appropriate environment.

The hinge region can take many different forms. Its design involves selection of amino acid residues (and a DNA fragment encoding them) which impart to the region of the fused protein about the cleavage site an appropriate polarity, charge distribution, and stereochemistry which, in the aqueous environment where the cleavage takes place, efficiently exposes the cleavage site to the cleavage agent in preference to other potential cleavage sites that may be present in the polypeptide, and/or to improve the kinetics of the cleavage reaction. In specific cases, the amino acids of the hinge are selected and assembled in sequence based on their known properties, and then the fused polypeptide sequence is expressed, tested, and altered for refinement.

The hinge region is free of cysteine. This enables the cleavage reaction to be conducted under conditions in which the protein assumes its tertiary conformation, and may be held in this conformation by intramolecular disulfide bonds. It has been discovered that in these conditions access of the protease to

potential cleavage sites which may be present within the target protein is hindered. The hinge region may comprise an amino acid sequence which includes one or more proline residues. This allows formation of a substantially unfolded molecular segment. Aspartic acid, glutamic acid, arginine, lysine, serine, and threonine residues maximize ionic interactions and may be present in amounts and/or in sequence which renders the moiety comprising the hinge water soluble.

The cleavage site preferably is immediately adjacent the Fv polypeptide chains and comprises one amino acid or a sequence of amino acids exclusive of any sequence found in the amino acid structure of the chains in the Fv. The cleavage site preferably is designed for unique or preferential cleavage by a specific selected agent. Endopeptidases are preferred, although non-enzymatic (chemical) cleavage agents may be used. Many useful cleavage agents, for instance, cyanogen bromide, dilute acid, trypsin, *Staphylococcus aureus* V-8 protease, post proline cleaving enzyme, blood coagulation Factor Xa, enterokinase, and renin, recognize and preferentially or exclusively cleave particular cleavage sites. One currently preferred cleavage agent is V-8 protease. The currently preferred cleavage site is a Glu residue. Other useful enzymes recognize multiple residues as a cleavage site, e.g., factor Xa (Ile-Glu-Gly-Arg) or enterokinase (Asp-Asp-Asp-Asp-Lys). The principles of this selective cleavage approach may also be used in the design of the linker and spacer sequences of the multifunctional constructs of the invention where an exciseable linker or selectively cleavable linker or spacer is desired.

#### Design of Synthetic V<sub>H</sub> and V<sub>L</sub> Mimics

FRs from the heavy and light chain murine anti-digoxin monoclonal 26-10 (Figures 4A and 4B) were encoded on the same DNAs with CDRs from the murine anti-lysozyme monoclonal glp-4 heavy chain (Figure 3 sequence 1) and light chain to produce V<sub>H</sub> (Figure 4C) and V<sub>L</sub> (Figure 4D) regions together defining a biosynthetic antibody binding site which is specific for lysozyme. Murine CDRs from both the heavy and light chains of monoclonal glp-4 were encoded on the same DNAs with FRs from the heavy and light chains of human myeloma antibody NEWM (Figures 4E and 4F). The resulting interspecies chimeric antibody binding domain has reduced immunogenicity in humans because of its human FRs, and specificity for lysozyme because of its murine CDRs.

A synthetic DNA was designed to facilitate CDR insertions into a human heavy chain FR and to facilitate empirical refinement of the resulting chimeric amino acid sequence. This DNA is depicted in Figure 5.

A synthetic, bifunctional FB-binding site protein was also designed at the DNA level, expressed, purified, renatured, and shown to bind specifically with a preselected antigen (digoxin) and Fc. The detailed primary structure of this construct is shown in Figure 6; its tertiary structure is illustrated schematically in Figure 2B.

Details of these and other experiments, and additional design principles on which the invention is based, are set forth below.

#### GENE DESIGN AND EXPRESSION

Given known variable region DNA sequences, synthetic V<sub>L</sub> and V<sub>H</sub> genes may be designed which encode native or near native FR and CDR amino acid sequences from an antibody molecule, each separated by unique restriction sites located as close to FR-CDR and CDR-FR borders as possible. Alternatively, genes may be designed which encode native FR sequences which are similar or identical to the FRs of an antibody molecule from a selected species, each separated by "dummy" CDR sequences containing strategically located restriction sites. These DNAs serve as starting materials for producing BABS, as the native or "dummy" CDR sequences may be excised and replaced with sequences encoding the CDR amino acids defining a selected binding site. Alternatively, one may design and directly synthesize native or near-native FR sequences from a first antibody molecule, and CDR sequences from a second antibody molecule. Any one of the V<sub>H</sub> and V<sub>L</sub> sequences described above may be linked together directly, via an amino acids chain or linker connecting the C-terminus of one chain with the N-terminus of the other.

These genes, once synthesized, may be cloned with or without additional DNA sequences coding for, e.g., an antibody constant region, enzyme, or toxin, or a leader peptide which facilitates secretion or intracellular stability of a fusion polypeptide. The genes then can be expressed directly in an appropriate host cell, or can be further engineered before expression by the exchange of FR, CDR, or "dummy" CDR sequences with new sequences. This manipulation is facilitated by the presence of the restriction sites which have been engineered into the gene at the FR-CDR and CDR-FR borders.

Figure 3 illustrates the general approach to designing a chimeric  $V_H$ ; further details of exemplary designs at the DNA level are shown in Figures 4A-4F. Figure 3, lines 1 and 2, show the amino acid sequences of the heavy chain variable region of the murine monoclonals glp-4 (anti-lysozyme) and 26-10 (anti-digoxin), including the four FR and three CDR sequences of each. Line 3 shows the sequence of a chimeric  $V_H$  which comprises 26-10 FRs and glp-4 CDRs. As illustrated, the hybrid protein of line 3 is identical to the native protein of line 2, except that 1) the sequence TFTNYYIHWLK has replaced the sequence IFTDFYMNWVR, 2) EWIGWIYPGNGNTKYNNFKG has replaced DYIGYISPYSGVTGYNQKFKG, 3) RYTHYYF has replaced GSSGNKWAM, and 4) A has replaced V as the sixth amino acid beyond CDR-2. These changes have the effect of changing the specificity of the 26-10  $V_H$  to mimic the specificity of glp-4. The Ala to Val single amino acid replacement within the relatively conserved framework region of 26-10 is an example of the replacement of an amino acid outside the hypervariable region made for the purpose of altering specificity by CDR replacement. Beneath sequence 3 of Figure 3, the restriction sites in the DNA encoding the chimeric  $V_H$  (see Figures 4A-4F) are shown which are disposed about the CDR-FR borders.

Lines 4 and 5 of Figure 3 represent another construct. Line 4 is the full length  $V_H$  of the human antibody NEWM. That human antibody may be made specific for lysozyme by CDR replacement as shown in line 5. Thus, for example, the segment TFTNYYIHWLK from glp-4 replaces TFSNDYYTWVR of NEWM, and its other CDRs are replaced as shown. This results in a  $V_H$  comprising a human framework with murine sequences determining specificity.

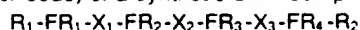
By sequencing any antibody, or obtaining the sequence from the literature, in view of this disclosure one skilled in the art can produce a BABS of any desired specificity comprising any desired framework region. Diagrams such as Figure 3 comparing the amino acid sequence are valuable in suggesting which particular amino acids should be replaced to determine the desired complementarity. Expressed sequences may be tested for binding and refined by exchanging selected amino acids in relatively conserved regions, based on observation of trends in amino acid sequence data and/or computer modeling techniques.

Significant flexibility in  $V_H$  and  $V_L$  design is possible because the amino acid sequences are determined at the DNA level, and the manipulation of DNA can be accomplished easily.

For example, the DNA sequence for murine  $V_H$  and  $V_L$  26-10 containing specific restriction sites flanking each of the three CDRs was designed with the aid of a commercially available computer program which performs combined reverse translation and restriction site searches ("RV.exe" by Compugene, Inc.). The known amino acid sequences for  $V_H$  and  $V_L$  26-10 polypeptides were entered, and all potential DNA sequences which encode those peptides and all potential restriction sites were analyzed by the program. The program can, in addition, select DNA sequences encoding the peptide using only codons preferred by *E. coli* if this bacterium is to be host expression organism of choice. Figures 4A and 4B show an example of program output. The nucleic acid sequences of the synthetic gene and the corresponding amino acids are shown. Sites of restriction endonuclease cleavage are also indicated. The CDRs of these synthetic genes are underlined.

The DNA sequences for the synthetic 26-10  $V_H$  and  $V_L$  are designed so that one or both of the restriction sites flanking each of the three CDRs are unique. A six base site (such as that recognized by Bsm I or BspM I) is preferred, but where six base sites are not possible, four or five base sites are used. These sites, if not already unique, are rendered unique within the gene by eliminating other occurrences within the gene without altering necessary amino acid sequences. Preferred cleavage sites are those that, once cleaved, yield fragments with sticky ends just outside of the boundary of the CDR within the framework. However, such ideal sites are only occasionally possible because the FR-CDR boundary is not an absolute one, and because the amino acid sequence of the FR may not permit a restriction site. In these cases, flanking sites in the FR which are more distant from the predicted boundary are selected.

Figure 5 discloses the nucleotide and corresponding amino acid sequence (shown in standard single letter code) of a synthetic DNA comprising a master framework gene having the generic structure:



where  $R_1$  and  $R_2$  are restricted ends which are to be ligated into a vector, and  $X_1$ ,  $X_2$ , and  $X_3$  are DNA sequences whose function is to provide convenient restriction sites for CDR insertion. This particular DNA has murine FR sequences and unique, 6-base restriction sites adjacent the FR borders so that nucleotide sequences encoding CDRs from a desired monoclonal can be inserted easily. Restriction endonuclease digestion sites are indicated with their abbreviations; enzymes of choice for CDR replacement are underscored. Digestion of the gene with the following restriction endonucleases results in 3' and 5' ends which can easily be matched up with and ligated to native or synthetic CDRs of desired specificity; KpnI and BstXI are used for ligation of CDR<sub>1</sub>; XbaI and DraI for CDR<sub>2</sub>; and BssHII and ClaI for CDR<sub>3</sub>.

## OLIGONUCLEOTIDE SYNTHESIS

The synthetic genes and DNA fragments designed as described above preferably are produced by assembly of chemically synthesized oligonucleotides. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

## CLONING OF SYNTHETIC OLIGONUCLEOTIDES

The blocks or the pairs of longer oligonucleotides may be cloned into *E. coli* using a suitable, e.g., pUC, cloning vector. Initially, this vector may be altered by single strand mutagenesis to eliminate residual six base altered sites. For example,  $V_H$  may be synthesized and cloned into pUC as five primary blocks spanning the following restriction sites: 1. EcoRI to first NarI site; 2. first NarI to XbaI; 3. XbaI to SalI; 4. SalI to NcoI; 5. NcoI to BamHI. These cloned fragments may then be isolated and assembled in several three-fragment ligations and cloning steps into the pUC8 plasmid. Desired ligations selected by PAGE are then transformed into, for example, *E. coli* strain JM83, and plated onto LB Ampicillin + Xgal plates according to standard procedures. The gene sequence may be confirmed by supercoil sequencing after cloning, or after subcloning into M13 via the dideoxy method of Sanger.

## PRINCIPLE OF CDR EXCHANGE

Three CDRs (or alternatively, four FRs) can be replaced per  $V_H$  or  $V_L$ . In simple cases, this can be accomplished by cutting the shuttle pUC plasmid containing the respective genes at the two unique restriction sites flanking each CDR or FR, removing the excised sequence, and ligating the vector with a native nucleic acid sequence or a synthetic oligonucleotide encoding the desired CDR or FR. This three part procedure would have to be repeated three times for total CDR replacement and four times for total FR replacement. Alternatively, a synthetic nucleotide encoding two consecutive CDRs separated by the appropriate FR can be ligated to a pUC or other plasmid containing a gene whose corresponding CDRs and FR have been cleaved out. This procedure reduces the number of steps required to perform CDR and/or FR exchange.

## EXPRESSION OF PROTEINS

The engineered genes can be expressed in appropriate prokaryotic hosts such as various strains of *E. coli*, and in eucaryotic hosts such as Chinese hamster ovary cell, murine myeloma, and human myeloma/transfectoma cells.

For example, if the gene is to be expressed in *E. coli*, it may first be cloned into an expression vector. This is accomplished by positioning the engineered gene downstream from a promoter sequence such as trp or tac, and a gene coding for a leader peptide. The resulting expressed fusion protein accumulates in refractile bodies in the cytoplasm of the cells, and may be harvested after disruption of the cells by French press or sonication. The refractile bodies are solubilized, and the expressed proteins refolded and cleaved by the methods already established for many other recombinant proteins.

If the engineered gene is to be expressed in myeloma cells, the conventional expression system for immunoglobulins, it is first inserted into an expression vector containing, for example, the Ig promoter, a secretion signal, immunoglobulin enhancers, and various introns. This plasmid may also contain sequences encoding all or part of a constant region, enabling an entire part of a heavy or light chain to be expressed. The gene is transfected into myeloma cells via established electroporation or protoplast fusion methods. Cells so transfected can express  $V_L$  or  $V_H$  fragments,  $V_{L2}$  or  $V_{H2}$  homodimers,  $V_L$ - $V_H$  heterodimers,  $V_H$ - $V_L$  or  $V_L$ - $V_H$  single chain polypeptides, complete heavy or light immunoglobulin chains, or portions thereof, each of which may be attached in the various ways discussed above to a protein region having another function (e.g., cytotoxicity).

Vectors containing a heavy chain V region (or V and C regions) can be cotransfected with analogous vectors carrying a light chain V region (or V and C regions), allowing for the expression of noncovalently associated binding sites (or complete antibody molecules).

In the examples which follow, a specific example of how to make a single chain binding site is disclosed, together with methods employed to assess its binding properties. Thereafter, a protein construct

having two functional domains is disclosed. Lastly, there is disclosed a series of additional targeted proteins which exemplify the invention.

#### I EXAMPLE OF CDR EXCHANGE AND EXPRESSION

5

The synthetic gene coding for murine  $V_H$  and  $V_L$  26-10 shown in Figures 4A and 4B were designed from the known amino acid sequence of the protein with the aid of Compugene, a software program. These genes, although coding for the native amino acid sequences, also contain non-native and often unique restriction sites flanking nucleic acid sequences encoding CDR's to facilitate CDR replacement as noted above.

10

Both the 3' and 5' ends of the large synthetic oligomers were designed to include 6-base restriction sites, present in the genes and the pUC vector. Furthermore, those restriction sites in the synthetic genes which were only suited for assembly but not for cloning the pUC were extended by "helper" cloning sites with matching sites in pUC.

15

Cloning of the synthetic DNA and later assembly of the gene is facilitated by the spacing of unique restriction sites along the gene. This allows corrections and modifications by cassette mutagenesis at any location. Among them are alterations near the 5' or 3' ends of the gene as needed for the adaptation to different expression vectors. For example, a PstI site is positioned near the 5' end of the  $V_H$  gene. Synthetic linkers can be attached easily between this site and a restriction site in the expression plasmid. These genes were synthesized by assembling oligonucleotides as described above using a Biosearch Model 8600 DNA Synthesizer. They were ligated to vector pUC8 for transformation of *E. coli*.

20

Specific CDRs may be cleaved from the synthetic  $V_H$  gene by digestion with the following pairs of restriction endonucleases: HpaI and BstXI for CDR<sub>1</sub>; XbaI and DraI for CDR<sub>2</sub>; and BanII and BstI for CDR<sub>3</sub>. After removal on one CDR, another CDR of desired specificity may be ligated directly into the restricted gene, in its place if the 3' and 5' ends of the restricted gene and the new CDR contain complementary single stranded DNA sequences.

25

In the present example, the three CDRs of each of murine  $V_H$  26-10 and  $V_L$  26-10 were replaced with the corresponding CDRs of glp-4. The nucleic acid sequences and corresponding amino acid sequences of the chimeric  $V_H$  and  $V_L$  genes encoding the FRs of 26-10 and CDRs of glp-4 are shown in Figures 4C and 4D. The positions of the restriction endonuclease cleavage sites are noted with their standard abbreviations. CDR sequences are underlined as are the restriction endonucleases of choice useful for further CDR replacement.

30

These genes were cloned into pUC8, a shuttle plasmid. To retain unique restriction sites after cloning, the  $V_H$ -like gene was spliced into the EcoRI and HindIII or BamHI sites of the plasmid.

35

Direct expression of the genes may be achieved in *E. coli*. Alternatively, the gene may be preceded by a leader sequence and expressed in *E. coli* as a fusion product by splicing the fusion gene into the host gene whose expression is regulated by interaction of a repressor with the respective operator. The protein can be induced by starvation in minimal medium and by chemical inducers. The  $V_H$ - $V_L$  biosynthetic 26-10 gene has been expressed as such a fusion protein behind the trp and lac promoters. The gene translation product of interest may then be cleaved from the leader in the fusion protein by e.g., cyanogen bromide degradation, tryptic digestion, mild acid cleavage, and/or digestion with factor Xa protease. Therefore, a shuttle plasmid containing a synthetic gene encoding a leader peptide having a site for mild acid cleavage, and into which has been spliced the synthetic BABS gene was used for this purpose. In addition, synthetic DNA sequences encoding a signal peptide for secretion of the processed target protein into the periplasm of the host cell can also be incorporated into the plasmid.

45

After harvesting the gene product and optionally releasing it from a fusion peptide, its activity as an antibody binding site and its specificity for glp-4 (lysozyme) epitope are assayed by established immunological techniques, e.g., affinity chromatography and radioimmunoassay. Correct folding of the protein to yield the proper three-dimensional conformation of the antibody binding site is prerequisite for its activity. This occurs spontaneously in a host such as a myeloma cell which naturally expresses immunoglobulin proteins. Alternatively, for bacterial expression, the protein forms inclusion bodies which, after harvesting, must be subjected to a specific sequence of solvent conditions (e.g., diluted 20 X from 8 M urea 0.1 M Tris-HCl pH 9 into 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (Hochman et al. (1976) Biochem. 15:2706-2710) to assume its correct conformation and hence its active form.

50

Figures 4E and 4F show the DNA and amino acid sequence of chimeric  $V_H$  and  $V_L$  comprising human FRs from NEWM and murine CDRs from glp-4. The CDRs are underlined, as are restriction sites of choice for further CDR replacement or empirically determined refinement.

55



These constructs also constitute master framework genes, this time constructed of human framework sequences. They may be used to construct BABS of any desired specificity by appropriate CDR replacement.

Binding sites with other specificities have also been designed using the methodologies disclosed herein. Examples include those having FRs from the human NEWM antibody and CDRs from murine 26-10 (Figure 9A), murine 26-10 FRs and G-loop CDRs (Figure 9B), FRs and CDRs from murine MOPC-315 (Figure 9C), FRs and CDRs from an anti-human carcinoembryonic antigen monoclonal antibody (Figure 9D), and FRs and CDRs 1, 2, and 3 from  $V_L$  and FRs and CDR 1 and 3 from the  $V_H$  of the anti-CEA antibody, with CDR 2 from a consensus immunoglobulin gene (Figure 9E).

## II. Model Binding Site:

The digoxin binding site of the IgG<sub>2a,k</sub> monoclonal antibody 26-10 has been analyzed by Mudgett-Hunter and colleagues (unpublished). The 26-10 V region sequences were determined from both amino acid sequencing and DNA sequencing of 26-10 H and L chain mRNA transcripts (D. Panka, J.N. & M.N.M., unpublished data). The 26-10 antibody exhibits a high digoxin binding affinity [ $K_D = 5.4 \times 10^9 \text{ M}^{-1}$ ] and has a well-defined specificity profile, providing a baseline for comparison with the biosynthetic binding sites mimicking its structure.

## Protein Design:

Crystallographically determined atomic coordinates for Fab fragments of 26-10 were obtained from the Brookhaven Data Bank. Inspection of the available three-dimensional structures of Fv regions within their parent Fab fragments indicated that the Euclidean distance between the C-terminus of the  $V_H$  domain and the N-terminus of the  $V_L$  domain is about 35 Å. Considering that the peptide unit length is approximately 3.8 Å, a 15 residue linker was selected to bridge this gap. The linker was designed so as to exhibit little propensity for secondary structure and not to interfere with domain folding. Thus, the 15 residue sequence (Gly-Gly-Gly-Gly-Ser)<sub>3</sub> was selected to connect the  $V_H$  carboxyl- and  $V_L$  amino-termini.

Binding studies with single chain binding sites having less than or greater than 15 residues demonstrate the importance of the prerequisite distance which must separate  $V_H$  from  $V_L$ ; for example, a (Gly-Ser)<sub>1</sub> linker does not demonstrate binding activity, and those with (Gly-Ser)<sub>5</sub> linkers exhibit very low activity compared to those with (Gly-Ser)<sub>3</sub> linkers.

## Gene Synthesis:

Design of the 744 base sequence for the synthetic binding site gene was derived from the Fv protein sequence of 26-10 by choosing codons frequently used in *E. coli*. The model of this representative synthetic gene is shown in Figure 8, discussed previously. Synthetic genes coding for the trp promoter-operator, the modified trp LE leader peptide (MLE), the sequence of which is shown in Figure 10A, and  $V_H$  were prepared largely as described previously. The gene coding for  $V_H$  was assembled from 46 chemically synthesized oligonucleotides, all 15 bases long, except for terminal fragments (13 to 19 bases) that included cohesive cloning ends. Between 8 and 15 overlapping oligonucleotides were enzymatically ligated into double stranded DNA, cut at restriction sites suitable for cloning (NarI, XbaI, SalI, SacII, SacI), purified by PAGE on 8% gels, and cloned in pUC which was modified to contain additional cloning sites in the polylinker. The cloned segments were assembled stepwise into the complete gene mimicking  $V_H$  by ligations in the pUC cloning vector.

The gene mimicking 26-10  $V_L$  was assembled from 12 long synthetic polynucleotides ranging in size from 33 to 88 base pairs, prepared in automated DNA synthesizers (Model 6500, Biosearch, San Rafael, CA; Model 380A, Applied Biosystems, Foster City, CA). Five individual double stranded segments were made out of pairs of long synthetic oligonucleotides spanning six-base restriction sites in the gene (AatII, BstEII, Ppnl, HindIII, BglII, and PstI). In one case, four long overlapping strands were combined and cloned. Gene fragments bounded by restriction sites for assembly that were absent from the pUC polylinker, such as AatII and BstEII, were flanked by EcoRI and BamHI ends to facilitate cloning.

The linker between  $V_H$  and  $V_L$ , encoding (Gly-Gly-Gly-Gly-Ser)<sub>3</sub>, was cloned from two long synthetic oligonucleotides, 54 and 62 bases long, spanning SacI and AatII sites, the latter followed by an EcoRI cloning end. The complete single chain binding site gene was assembled from the  $V_H$ ,  $V_L$ , and linker genes to produce a construct, corresponding to aspartyl-prolyl- $V_H$ -(linker)- $V_L$ , flanked by EcoRI and PstI restriction sites.

The *trp* promoter-operator, starting from its *Ssp*I site, was assembled from 12 overlapping 15 base oligomers, and the MLE leader gene was assembled from 24 overlapping 15 base oligomers. These were cloned and assembled in pUC using the strategy of assembly sites flanked by cloning sites. The final expression plasmid was constructed in the pBR322 vector by a 3-part ligation using the sites *Ssp*I, *Eco*RI, and *Pst*I (see Figure 10B). Intermediate DNA fragments and assembled genes were sequenced by the dideoxy method.

#### Fusion Protein Expression:

Single-chain protein was expressed as a fusion protein. The MLE leader gene (Fig. 10A) was derived from *E. coli trp* LE sequence and expressed under the control of a synthetic *trp* promoter and operator. *E. coli* strain JM83 was transformed with the expression plasmid and protein expression was induced in M9 minimal medium by addition of indoleacrylic acid (10  $\mu$ g/ml) at a cell density with  $A_{600} = 1$ . The high expression levels of the fusion protein resulted in its accumulation as insoluble protein granules, which were harvested from cell paste (Figure 11, Lane 1).

#### Fusion Protein Cleavage:

The MLE leader was removed from the binding site protein by acid cleavage of the Asp-Pro peptide bond engineered at the junction of the MLE and binding site sequences. The washed protein granules containing the fusion protein were cleaved in 6 M guanidine-HCl + 10% acetic acid, pH 2.5, incubated at 37°C for 96 hrs. The reaction was stopped through precipitation by addition of a 10-fold excess of ethanol with overnight incubation at -20°C, followed by centrifugation and storage at -20°C until further purification (Figure 11, Lane 2).

#### Protein Purification:

The acid cleaved binding site was separated from remaining intact fused protein species by chromatography on DEAE® cellulose. The precipitate obtained from the cleavage mixture was redissolved in 6 M guanidine-HCl + 0.2 M Tris-HCl, pH 8.2, + 0.1 M 2-mercaptoethanol and dialyzed exhaustively against 6 M urea + 2.5 mM Tris-HCl, pH 7.5, + 1 mM EDTA. 2-Mercaptoethanol was added to a final concentration of 0.1 M, the solution was incubated for 2 hrs at room temperature and loaded onto a 2.5 X 45 cm column of DEAE cellulose (Whatman DE 52), equilibrated with 6 M urea + 2.5 mM Tris-HCl + 1 mM EDTA, pH 7.5. The intact fusion protein bound weakly to the DE 52 column such that its elution was retarded relative to that of the binding protein. The first protein fractions which eluted from the column after loading and washing with urea buffer contained BABS protein devoid of intact fusion protein. Later fractions contaminated with some fused protein were pooled, rechromatographed on DE 52, and recovered single chain binding protein combined with other purified protein into a single pool (Figure 11, Lane 3).

#### Refolding:

The 26-10 binding site mimic was refolded as follows: the DE 52 pool, disposed in 6 M urea + 2.5 mM Tris-HCl + 1 mM EDTA, was adjusted to pH 8 and reduced with 0.1 M 2-mercaptoethanol at 37°C for 90 min. This was diluted at least 100-fold with 0.01 M sodium acetate, pH 5.5, to a concentration below 10  $\mu$ g/ml and dialyzed at 4°C for 2 days against acetate buffer.

#### Affinity Chromatography:

Purification of active binding protein by affinity chromatography at 4°C on a ouabain-amine-Sepharose® column was performed. The dilute solution of refolded protein was loaded directly onto a pair of tandem columns, each containing 3 ml of resin equilibrated with the 0.01 M acetate buffer, pH 5.5. The columns were washed individually with an excess of the acetate buffer, and then by sequential additions of 5 ml each of 1 M NaCl, 20 mM ouabain, and 3 M potassium thiocyanate dissolved in the acetate buffer, interspersed with acetate buffer washes. Since digoxin binding activity was still present in the eluate, the eluate was pooled and concentrated 20-fold by ultrafiltration (PM 10 membrane, 200 ml concentrator; Amicon), reapplied to the affinity columns, and eluted as described. Fractions with significant absorbance at 280 nm were pooled and dialyzed against PBSA or the above acetate buffer. The amounts of protein in the DE 52 and ouabain-Sepharose® pools were quantitated by amino acid analysis following dialysis against

0.01 M acetate buffer. The results are shown below in Table 1.

TABLE 1

Estimated Yields of BABS Protein During Purification				
Step	Wet wt. Per l	mg protein	Cleavage yield (%) prior step	Yield relative to fusion protein
Cell paste	12.0 g	1440.0 mg <sup>a</sup>	-	-
Fusion protein Granules	2.3 g	480.0 mg <sup>a,b</sup>	100.0%	100.0%
Acid Cleavage-DE 52 pool		144.0 mg	38.0 <sup>c</sup>	38.0 <sup>c</sup>
Ouabain-Sepharose pool		18.1 mg	12.6 <sup>d</sup>	4.7 <sup>e</sup>

<sup>a</sup>Determined by Lowry protein analysis

<sup>b</sup>Determined by absorbance measurements

<sup>c</sup>Determined by amino acid analysis

<sup>d</sup>Calculated from the amount of BABS protein specifically eluted from ouabain-Sepharose® relative to that applied to the resin; values were determined by amino acid analysis

<sup>e</sup>Percentage yield calculated on a molar basis

#### Sequence Analysis of Gene and Protein:

The complete gene was sequenced in both directions using the dideoxy method of Sanger which confirmed the gene was correctly assembled. The protein sequence was also verified by protein sequencing. Automated Edman degradation was conducted on intact protein (residues 1-40), as well as on two major CNBr fragments (residues 108-129 and 140-159) with a Model 470A gas phase sequencer equipped with a Model 120A on-line phenylthiohydantoin-amino acid analyzer (Applied Biosystems, Foster City, CA). Homogeneous binding protein fractionated by SDS-PAGE and eluted from gel strips with water, was treated with a 20,000-fold excess of CNBr, in 1% trifluoroacetic acid-acetonitrile (1:1), for 12 hrs at 25° (in the dark). The resulting fragments were separated by SDS-PAGE and transferred electrophoretically onto an Immobilon membrane (Millipore, Bedford, MA), from which stained bands were cut out and sequenced.

#### Specificity Determination:

Specificities of anti-digoxin 26-10 Fab and the BABS were assessed by radioimmunoassay. Wells of microtiter plates were coated with affinity-purified goat anti-murine Fab fragment (ICN ImmunoBiologicals, Lisle, IL) at 10 µg/ml in PBSA overnight at 4°C. After the plates were washed and blocked with 1% horse serum in PBSA, solutions (50 µl) containing 26-10 Fab or the BABS in either PBSA or 0.01 M sodium acetate at pH 5.5 were added to the wells and incubated 2-3 hrs at room temperature. After unbound antibody fragment was washed from the wells, 25 µl of a series of concentrations of cardiac glycosides (10<sup>-4</sup> to 10<sup>-11</sup> M in PBSA) were added. The cardiac glycosides tested included digoxin, digitoxin, digoxigenin, digitoxigenin, gitoxin, ouabain, and acetyl strophanthidin. After the addition of <sup>125</sup>I-digoxin (25 µl, 50,000 cpm; Cambridge Diagnostics, Billerica, MA) to each well, the plates were incubated overnight at 4°C, washed and counted. The inhibition curves are plotted in Figure 12. The relative affinities for each digoxin analogue were calculated by dividing the concentration of each analogue at 50% inhibition by the concentration of digoxin (or digoxigenin) that gave 50% inhibition. There is a displacement of inhibition curves for the BABS to lower glycoside concentrations than observed for 26-10 Fab, because less active BABS than 26-10 Fab was bound to the plate. When 0.25 M urea was added to the BABS in 0.01 M sodium acetate, pH 5.5, more active sFv was bound to the goat anti-murine Fab coating on the plate. This caused the BABS inhibition curves to shift toward higher glycoside concentrations, closer to the position of those for 26-10 Fab, although maintaining the relative positions of curves for sFv obtained in acetate buffer alone. The results, expressed as normalized concentration of inhibitor giving 50% inhibition of <sup>125</sup>I-digoxin binding, are shown in Table 2.

TABLE 2

26-10 Antibody Species	Normalizing Glycoside	D	DG	DO	DOG	A-S	G	O
Fab	Digoxin	1.0	1.2	0.9	1.0	1.3	9.6	15
	Digoxigenin	0.9	1.0	0.8	0.9	1.1	8.1	13
BABS	Digoxin	1.0	7.3	2.0	2.6	5.9	62	150
	Digoxigenin	0.1	1.0	0.3	0.4	0.8	8.5	21
D = Digoxin DG = Digoxigenin DO = Digitoxin DOG = Digitoxigenin A-S = Acetyl Strophanthidin G = Gitoxin O = Ouabain								

#### Affinity Determination:

Association constants were measured by equilibrium binding studies. In immunoprecipitation experiments, 100  $\mu$ l of  $^3$ H-digoxin (New England Nuclear, Billerica, MA) at a series of concentrations ( $10^{-7}$  M to  $10^{-11}$  M) were added to 100  $\mu$ l of 26-10 Fab or the BABS at a fixed concentration. After 2-3 hrs of incubation at room temperature, the protein was precipitated by the addition of 100  $\mu$ l goat antiserum to murine Fab fragment (ICN ImmunoBiologicals), 50  $\mu$ l of the IgG fraction of rabbit anti-goat IgG (ICN ImmunoBiologicals), and 50  $\mu$ l of a 10% suspension of protein A-Sepharose® (Sigma). Following 2 hrs at 4°C, bound and free antigen were separated by vacuum filtration on glass fiber filters (Vacuum Filtration Manifold, Millipore, Bedford, MA). Filter disks were then counted in 5 ml of scintillation fluid with a Model 1500 Tri-Carb Liquid Scintillation Analyzer (Packard, Sterling, VA). The association constants,  $K_o$ , were calculated from Scatchard analyses of the untransformed radioligand binding data using LIGAND, a non-linear curve fitting program based on mass action.  $K_o$ s were also calculated by Sips plots and binding isotherms shown in Figure 13A for the BABS and 13B for the Fab. For binding isotherms, data are plotted as the concentration of digoxin bound versus the log of the unbound digoxin concentration, and the dissociation constant is estimated from the ligand concentration at 50% saturation. These binding data are also plotted in linear form as Sips plots (inset), having the same abscissa as the binding isotherm but with the ordinate representing  $\log r/(n-r)$ , defined below. The average intrinsic association constant ( $K_o$ ) was calculated from the modified Sips equation (39),  $\log (r/n-r) = a \log C - a \log K_o$ , where  $r$  equals moles of digoxin bound per mole of antibody at an unbound digoxin concentration equal to  $C$ ;  $n$  is the number of moles of digoxin bound at saturation of the antibody binding site, and  $a$  is an index of heterogeneity which describes the distribution of association constants about the average intrinsic association constant  $K_o$ . Least squares linear regression analysis of the data indicated correlation coefficients for the lines obtained were 0.96 for the BABS and 0.99 for 26-10 Fab. A summary of the calculated association constants are shown below in Table 3.

TABLE 3

Method of Data Analysis	Association Constant, $K_o$	
	$K_o$ (BABS), $M^{-1}$	$K_o$ (Fab), $M^{-1}$
Scatchard plot	$(3.2 \pm 0.9) \times 10^7$	$(1.9 \pm 0.2) \times 10^8$
Sips plot	$2.6 \times 10^7$	$1.8 \times 10^8$
Binding isotherm	$5.2 \times 10^7$	$3.3 \times 10^8$

### III. Synthesis of a Multifunctional Protein

A nucleic acid sequence encoding the single chain binding site described above was fused with a sequence encoding the FB fragment of protein A as a leader to function as a second active region. As a spacer, the native amino acids comprising the last 11 amino acids of the FB fragment bonded to an Asp-Pro dilute acid cleavage site was employed. The FB binding domain of the FB consists of the immediately preceding 43 amino acids which assume a helical configuration (see Fig. 2B).

The gene fragments are synthesized using a Biosearch DNA Model 8600 Synthesizer as described above. Synthetic oligonucleotides are cloned according to established protocol described above using the pUC8 vector transfected into *E. coli*. The completed fused gene set forth in Figure 6A is then expressed in *E. coli*.

After sonication, inclusion bodies were collected by centrifugation, and dissolved in 6 M guanidine hydrochloride (GuHCl), 0.2 M Tris, and 0.1 M 2-mercaptoethanol (BME), pH 8.2. The protein was denatured and reduced in the solvent overnight at room temperature. Size exclusion chromatography was used to purify fusion protein from the inclusion bodies. A Sepharose 4B column (1.5 X 80 cm) was run in a solvent of 6 M GuHCl and 0.01 M NaOAc, pH 4.75. The protein solution was applied to the column at room temperature in 0.5-1.0 ml amounts. Fractions were collected and precipitated with cold ethanol. These were run on SDS gels, and fractions rich in the recombinant protein (approximately 34,000 D) were pooled. This offers a simple first step for cleaning up inclusion body preparations without suffering significant proteolytic degradation.

For refolding, the protein was dialyzed against 100 ml of the same GuHCl-Tris-BME solution, and dialysate was diluted 11-fold over two days to 0.55 M GuHCl, 0.01 M Tris, and 0.01 M BME. The dialysis sacks were then transferred to 0.01 M NaCl, and the protein was dialyzed exhaustively before being assayed by RIA's for binding of <sup>125</sup>I-labelled digoxin. The refolding procedure can be simplified by making a rapid dilution with water to reduce the GuHCl concentration to 1.1 M, and then dialyzing against phosphate buffered saline (0.15 M NaCl, 0.05 M potassium phosphate, pH 7, containing 0.03% NaN<sub>3</sub>), so that it is free of any GuHCl within 12 hours. Product of both types of preparation showed binding activity, as indicated in Figure 7A.

#### Demonstration of Bifunctionality:

This protein with an FB leader and a fused BABS is bifunctional; the BABS can bind the antigen and the FB can bind the Fc regions of immunoglobulins. To demonstrate this dual and simultaneous activity several radioimmunoassays were performed.

Properties of the binding site were probed by a modification of an assay developed by Mudgett-Hunter et al. (J. Immunol. (1982) 129:1165-1172; Molec. Immunol. (1985) 22:477-488), so that it could be run on microtiter plates as a solid phase sandwich assay. Binding data were collected using goat anti-murine Fab antisera (gAmFab) as the primary antibody that initially coats the wells of the plate. These are polyclonal antisera which recognize epitopes that appear to reside mostly on framework regions. The samples of interest are next added to the coated wells and incubated with the gAmFab, which binds species that exhibit appropriate antigenic sites. After washing away unbound protein, the wells are exposed to <sup>125</sup>I-labelled (radioiodinated) digoxin conjugates, either as <sup>125</sup>I-dig-BSA or <sup>125</sup>I-dig-lysine.

The data are plotted in Figure 7A, which shows the results of a dilution curve experiment in which the parent 26-10 antibody was included as a control. The sites were probed with <sup>125</sup>I-dig-BSA as described above, with a series of dilutions prepared from initial stock solutions, including both the slowly refolded (1) and fast diluted/quickly refolded (2) single chain proteins. The parallelism between all three dilution curves indicates that gAmFab binding regions on the BABS molecule are essentially the same as on the Fv of authentic 26-10 antibody, i.e., the surface epitopes appear to be the same for both proteins.

The sensitivity of these assays is such that binding affinity of the Fv for digoxin must be at least 10<sup>6</sup>. Experimental data on digoxin binding yielded binding constants in the range of 10<sup>8</sup> to 10<sup>9</sup> M<sup>-1</sup>. The parent 26-10 antibody has an affinity of 5.4 X 10<sup>9</sup> M<sup>-1</sup>. Inhibition assays also indicate the binding of <sup>125</sup>I-dig-lysine, and can be inhibited by unlabelled digoxin, digoxigenin, digitoxin, digitoxigenin, gitoxin, acetyl strophanthidin, and ouabain in a way largely parallel to the parent 26-10 Fab. This indicates that the specificity of the biosynthetic protein is substantially identical to the original monoclonal.

In a second type of assay, Digoxin-BSA is used to coat microtiter plates. Renatured BABS (FB-BABS) is added to the coated plates so that only molecules that have a competent binding site can stick to the plate. <sup>125</sup>I-labelled rabbit IgG (radioligand) is mixed with bound FB-BABS on the plates. Bound radioactivity reflects the interaction of IgG with the FB domain of the BABS, and the specificity of this binding is

demonstrated by its inhibition with increasing amounts of FB, Protein A, rabbit IgG, IgG2a, and IgG1, as shown in Figure 7B.

The following species were tested in order to demonstrate authentic binding: unlabelled rabbit IgG and IgG2a monoclonal antibody (which binds competitively to the FB domain of the BABS); and protein A and FB (which bind competitively to the radioligand). As shown in Figure 7B, these species are found to completely inhibit radioligand binding, as expected. A monoclonal antibody of the IgG1 subclass binds poorly to the FB, as expected, inhibiting only about 34% of the radioligand from binding. These data indicate that the BABS domain and the FB domain have independent activity.

#### 10 IV. OTHER CONSTRUCTS

Other BABS-containing protein constructed according to the invention expressible in *E. coli* and other host cells as described above are set forth in the drawing. These proteins may be bifunctional or multifunctional. Each construct includes a single chain BABS linked via a spacer sequence to an effector molecule comprising amino acids encoding a biologically active effector protein such as an enzyme, receptor, toxin, or growth factor. Some examples of such constructs shown in the drawing include proteins comprising epidermal growth factor (EGF) (Figure 15A), streptavidin (Figure 15B), tumor necrosis factor (TNF) (Figure 15C), calmodulin (Figure 15D) the beta chain of platelet derived growth factor (B-PDGF) (15E) ricin A (15F), interleukin 2 (15G) and FB dimer (15H). Each is used as a trailer and is connected to a preselected BABS via a spacer (Gly-Ser-Gly) encoded by DNA defining a BamHI restriction site. Additional amino acids may be added to the spacer for empirical refinement of the construct if necessary by opening up the Bam HI site and inserting an oligonucleotide of a desired length having BamHI sticky ends. Each gene also terminates with a PstI site to facilitate insertion into a suitable expression vector.

The BABS of the EGF and PDGF constructs may be, for example, specific for fibrin so that the EGF or PDGF is delivered to the site of a wound. The BABS for TNF and ricin A may be specific to a tumor antigen, e.g., CEA, to produce a construct useful in cancer therapy. The calmodulin construct binds radioactive ions and other metal ions. Its BABS may be specific, for example, to fibrin or a tumor antigen, so that it can be used as an imaging agent to locate a thrombus or tumor. The streptavidin construct binds with biotin with very high affinity. The biotin may be labeled with a remotely detectable ion for imaging purposes. Alternatively, the biotin may be immobilized on an affinity matrix or solid support. The BABS-streptavidin protein could then be bound to the matrix or support for affinity chromatography or solid phase immunoassay. The interleukin-2 construct could be linked, for example, to a BABS specific for a T-cell surface antigen. The FB-FB dimer binds to Fc, and could be used with a BABS in an immunoassay or affinity purification procedure linked to a solid phase through immobilized immunoglobulin.

Figure 14 exemplifies a multifunctional protein having an effector segment as a leader. It comprises an FB-FB dimer linked through its C-terminal via an Asp-Pro dipeptide to a BABS of choice. It functions in a way very similar to the construct of Fig. 15H. The dimer binds avidly to the Fc portion of immunoglobulin. This type of construct can accordingly also be used in affinity chromatography, solid phase immunoassay, and in therapeutic contexts where coupling of immunoglobulins to another epitope is desired.

In view of the foregoing, it should be apparent that the invention is unlimited with respect to the specific types of BABS and effector proteins to be linked. Accordingly, other embodiments are within the following claims.

#### Claims

1. A single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques, said protein comprising:
  - a biosynthetic antibody binding site capable of binding to a preselected antigenic determinant and comprising an amino acid sequence homologous with the sequence of a variable region of an immunoglobulin molecule capable of binding said preselected antigenic determinant,
  - a first biofunctional domain comprising a polypeptide selected from the group consisting of effector proteins having a conformation suitable for biological activity in mammals, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support, and
  - a first polypeptide linker disposed between said binding site and said first biofunctional domain, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and which defines a polypeptide which connects the C-terminal end of said binding site and the N-terminal end of said first biofunctional domain or the N-terminal end of said binding site and the C-terminal end of said

first biofunctional domain, whereupon said binding protein assumes a conformation suitable for binding and said first biofunctional domain assumes a conformation suitable for biological activity, sequestering an ion, or selectively binding a solid support.

- 5 2. The protein of claim 1 wherein said binding site comprises at least two binding domains peptide bonded by a second polypeptide linker disposed between said domains, wherein said second polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids and which defines a polypeptide of a length sufficient to span the distance between the C-terminal end of one of said binding domains and the N-terminal end of the other of said binding domains when said binding protein  
10 assumes a conformation suitable for binding when disposed in aqueous solution.
3. The protein of claim 2 wherein the amino acid sequence of each of said binding domains comprises a set of CDRs interposed between a set of FRs, each of which is respectively homologous with CDRs and FRs from a said variable region of an immunoglobulin molecule capable of binding said  
15 preselected antigenic determinant.
4. The protein of claim 2 wherein at least one of said binding domains comprises a said set of CDRs homologous with the CDRs in a first immunoglobulin and a set of FRs homologous with the FRs in a second, distinct immunoglobulin.  
20
5. The protein of claim 1 or 2 wherein said effector protein is an enzyme, toxin, receptor, binding site, biosynthetic antibody binding site, growth factor, cell-differentiation factor, lymphokine, cytokine, hormone, a remotely detectable moiety, or anti-metabolite.
- 25 6. The protein of claim 1 or 2 wherein said sequence capable of sequestering an ion is calmodulin, methallothionein, a fragment thereof, or an amino acid sequence rich in at least one of glutamic acid, aspartic acid, lysine, and arginine.
- 30 7. The protein of claim 1 or 2 wherein said polypeptide sequence capable of selective binding to a solid support is a positively or negatively charged amino acid sequence, a cysteine-containing amino acid sequence, streptavidin, or a fragment of Staphylococcus protein A.
8. The protein of claim 2 comprising a plurality of biosynthetic antibody binding sites, each binding site comprising two said binding domains connected by a polypeptide linker.  
35
9. A DNA encoding the protein of claim 1.
10. A host cell harboring and capable of expressing the DNA of claim 9.
- 40 11. A single polypeptide chain comprising: a pair of polypeptide domains together defining a site for binding a preselected antigen, and being joined through the C-terminus of one to the N-terminus of the other by a polypeptide linker, wherein the amino acid sequence of each of said polypeptide domains mimics an immunoglobulin variable region, and at least one said domain comprises:  
a set of CDR amino acid sequences together defining a recognition site for said preselected  
45 antigen, wherein said CDR sequences are non-human sequences,  
a set of FR amino acid sequences linked to said set of CDR sequences, wherein said FR amino acid sequences are homologous to sequences obtained from a human immunoglobulin, and  
said linked sets of CDR and FR amino acid sequences together defining a chimeric binding domain which, when disposed in aqueous solution, assumes a tertiary structure suitable for immunological  
50 binding with said preselected antigen.
12. A single polypeptide chain comprising: a pair of polypeptide domains defining a site for binding a preselected antigen joined by a polypeptide linker spanning the distance between the C-terminal of one to the N-terminal of the other, wherein the amino acid sequence of at least one of said polypeptide  
55 domains comprises a recombinant variable region comprising:  
a set of CDR amino acid sequences together defining a recognition site for said preselected antigen, wherein said CDR sequences are homologous to sequences obtained from a first immunoglobulin.

a set of FR amino acid sequences linked to said set of CDR sequences, wherein said FR amino acid sequences are homologous to sequences obtained from a second immunoglobulin, and

said linked sets of CDR and Fr amino acid sequences together defining a chimeric single chain variable region binding polypeptide which, when disposed in aqueous solution, assumes a tertiary structure suitable for immunological binding with said preselected antigen.

13. The polypeptide chain of claim 12 wherein

the FR sequences are homologous with sequences obtained from a human immunoglobulin and the CDR sequences are homologous with sequences obtained from a murine immunoglobulin.

14. The polypeptide chain of claim 11 or 12 wherein said pair of polypeptide domains are peptide bonded to a biologically active domain via a second polypeptide linker disposed between said binding polypeptide and said biologically active domain, wherein said second polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids, which together assume an unstructured polypeptide configuration in aqueous solution, and connect the C-terminal end of said binding site and the N-terminal end of said biologically active domain or the N-terminal end of said binding site and the C-terminal end of said biologically active domain, whereupon said binding polypeptide assumes a conformation suitable for binding and said biologically active domain assumes a conformation suitable for biological activity, sequestering an ion, or selectively binding a solid support.

## Patentansprüche

1. Einzelkettiges multifunktionelles biosynthetisches Protein, das durch ein durch rekombinante DNA-Techniken erhaltenes Einzelgen exprimiert wird, wobei das Protein aufweist:

eine biosynthetische Antikörperbindungsstelle, die an eine vorgewählte antigene Determinante binden kann und eine Aminosäuresequenz aufweist, die homolog zu der Sequenz einer variablen Region eines Immunglobulinmoleküls ist, das die vorgewählte antigene Determinante binden kann,

eine erste biofunktionelle Domäne, die ein Polypeptid aufweist, das unter Effektorproteinen mit einer für biologische Aktivität in Säugern geeigneten Konformation, Aminosäuresequenzen, die ein Ion maskieren können, und Aminosäuresequenzen, die selektiv an einen festen Träger binden können, ausgewählt ist und

einen ersten Polypeptidlinker, der zwischen der Bindungsstelle und der ersten biofunktionellen Domäne angeordnet ist, wobei der Polypeptidlinker mehrere hydrophile, peptidgebundene Aminosäuren aufweist und ein Polypeptid definiert, welches das C-terminale Ende der Bindungsstelle und das N-terminale Ende der ersten biofunktionellen Domäne oder das N-terminale Ende der Bindungsstelle und das C-terminale Ende der ersten biofunktionellen Domäne verbindet, wodurch das Bindungsprotein eine zum Binden geeignete Konformation annimmt und die erste biofunktionelle Domäne eine für die biologische Aktivität, Maskierung eines Ions oder selektive Bindung eines festen Trägers geeignete Konformation annimmt.

2. Protein nach Anspruch 1, wobei die Bindungsstelle mindestens zwei Bindungsdomänen aufweist, die durch einen zweiten Polypeptidlinker peptidgebunden sind, der zwischen den Domänen angeordnet ist, wobei der zweite Polypeptidlinker mehrere hydrophile peptidgebundene Aminosäuren aufweist und ein Polypeptid mit einer ausreichenden Länge zur Überbrückung des Abstandes zwischen dem C-terminalen Ende einer der Bindungsdomänen und dem N-terminalen Ende der anderen der Bindungsdomänen definiert, wenn das Bindungsprotein eine zur Bindung geeignete Konformation einnimmt, wenn es in wässrige Lösung gebracht wird.

3. Protein nach Anspruch 2, wobei die Aminosäuresequenz jeder der Bindungsdomänen einen Satz von CDRs aufweist, die zwischen einem Satz von FRs angeordnet sind, welche jeweils entsprechend homolog zu CDRs und FRs einer variablen Region eines Immunglobulinmoleküls sind, das die vorgewählte antigene Determinante binden kann.

4. Protein nach Anspruch 2, wobei mindestens eine der Bindungsdomänen einen Satz von CDRs, die zu den CDRs eines ersten Immunglobulins homolog sind, und einen Satz von FRs, die zu den FRs eines zweiten, verschiedenen Immunglobulins homolog sind, aufweist.



5. Protein nach Anspruch 1 oder 2, wobei es sich bei dem Effektorprotein um ein Enzym, ein Toxin, einen Rezeptor, eine Bindungsstelle, eine biosynthetische Antikörperbindungsstelle, einen Wachstumsfaktor, einen Zelldifferenzierungsfaktor, ein Lymphokin, ein Cytokin, ein Hormon, einen indirekt nachweisbaren Rest oder einen Antimetaboliten handelt.
- 5 6. Protein nach Anspruch 1 oder 2, wobei es sich bei der ein Ion maskierenden Sequenz um Calmodulin, Methallothionein, ein Fragment davon oder um eine Aminosäuresequenz, die zumindest reich an einer der Aminosäuren Glutaminsäure, Asparaginsäure, Lysin und Arginin ist, handelt.
- 10 7. Protein nach Anspruch 1 oder 2, wobei es sich bei der selektiv an einen festen Träger bindenden Polypeptidsequenz um eine positiv oder negativ geladene Aminosäuresequenz, eine cysteinhaltige Aminosäuresequenz, Streptavidin oder ein Fragment von Protein A aus Staphylococcus handelt.
8. Protein nach Anspruch 2, das eine Vielzahl von biosynthetischen Antikörperbindungsstellen aufweist, wobei jede Bindungsstelle zwei der durch einen Polypeptidlinker verbunden Bindungsdomänen aufweist.
- 15 9. DNA, die das Protein nach Anspruch 1 codiert.
- 20 10. Wirtszelle, welche die DNA nach Anspruch 9 enthält und exprimieren kann.
11. Polypeptideinzelkette, die aufweist:  
ein Paar Polypeptiddomänen, die zusammen eine Bindungsstelle für ein vorgewähltes Antigen definieren und über einen Polypeptidlinker mit dem C-Terminus der einen an den N-Terminus der anderen gebunden sind, wobei die Aminosäuresequenz jeder der Polypeptiddomänen eine variable Region eines Immunglobulins nachahmt und mindestens eine der Domänen aufweist:  
25 einen Satz CDR-Aminosäuresequenzen, die zusammen eine Erkennungsstelle für das vorgewählte Antigen definieren, wobei es sich bei den CDR-Sequenzen um nichtmenschliche Sequenzen handelt, einen Satz FR-Aminosäuresequenzen, die an den Satz CDR-Sequenzen gebunden sind, wobei die FR-Aminosäuresequenzen zu aus einem menschlichen Immunglobulin erhaltenen Sequenzen homolog sind,  
30 wobei die gebundenen Sätze CDR- und FR-Aminosäuresequenzen zusammen eine chimäre Bindungsdomäne definieren, die in wässriger Lösung eine zur immunologischen Bindung mit dem vorgewählten Antigen geeignete Tertiärstruktur annimmt.
- 35 12. Polypeptideinzelkette, die aufweist:  
ein Paar Polypeptiddomänen, die eine Bindungsstelle für ein vorgewähltes Antigen definieren und durch einen Polypeptidlinker verbunden sind, der den Abstand zwischen dem C-Terminus der einen und dem N-Terminus der anderen überbrückt, wobei die Aminosäuresequenz mindestens einer der Polypeptiddomänen eine rekombinierte variable Region enthält, die aufweist:  
40 einen Satz CDR-Aminosäuresequenzen, die zusammen eine Erkennungsstelle für das vorgewählte Antigen definieren, wobei die CDR-Sequenzen zu aus einem ersten Immunglobulin erhaltenen Sequenzen homolog sind,  
einen Satz FR-Aminosäuresequenzen, die an den Satz CDR-Sequenzen gebunden sind, wobei die FR-Aminosäuresequenzen zu aus einem zweiten Immunglobulin erhaltenen Sequenzen homolog sind,  
45 wobei die gebundenen Sätze CDR- und FR-Aminosäuresequenzen zusammen ein chimäres Einzelkettenbindungspolypeptid mit variabler Region definieren, das in wässriger Lösung eine zur immunologischen Bindung mit dem vorgewählten Antigen geeignete Tertiärstruktur annimmt.
- 50 13. Polypeptidkette nach Anspruch 12, wobei die FR-Sequenzen zu aus einem menschlichen Immunglobulin erhaltenen Sequenzen homolog sind und die CDR-Sequenzen zu aus einem Mäuse-Immunglobulin erhaltenen Sequenzen homolog sind.
14. Polypeptidkette nach Anspruch 11 oder 12, wobei das Paar Polypeptiddomänen an eine biologisch aktive Domäne peptidgebunden ist, und zwar über einen zweiten Polypeptidlinker, der zwischen dem Bindungspolypeptid und der biologisch aktiven Domäne angeordnet ist, wobei der zweite Polypeptidlinker mehrere hydrophile peptidgebundene Aminosäuren aufweist, die zusammen in wässriger Lösung eine unstrukturierte Polypeptidkonfiguration annehmen, und das C-terminale Ende der Bindungsstelle
- 55

und das N-terminale Ende der biologisch aktiven Domäne oder das N-terminale Ende der Bindungsstelle und das C-terminale Ende der biologisch aktiven Domäne verbindet, wodurch das Bindungspolypeptid eine zur Bindung geeignete Konformation annimmt und die biologisch aktive Domäne eine für die biologische Aktivität, zur Maskierung eines Ions oder zur selektiven Bindung eines festen Trägers geeignete Konformation annimmt.

## Revendications

1. Une protéine biosynthétique multifonctionnelle à chaîne unique, exprimée à partir d'un gène unique dérivé par des techniques d'ADN recombinant, ladite protéine comprenant:
  - un site de liaison d'un anticorps biosynthétique capable de se lier à un déterminant antigénique présélectionné et comprenant une séquence d'acides aminés homologue à la séquence d'une région variable d'une molécule d'immunoglobuline capable de lier ledit déterminant antigénique présélectionné,
  - un premier domaine biofonctionnel comprenant un polypeptide sélectionné dans le groupe consistant en des protéines effectrices ayant une conformation appropriée pour une activité biologique chez des mammifères, des séquences d'acides aminés capables de séquestrer un ion et des séquences d'acides aminés capables de se lier sélectivement à un support solide, et
  - un premier polypeptide de jonction disposé entre ledit site de liaison et ledit premier domaine biofonctionnel, où ledit polypeptide de jonction comprend des acides aminés multiples, hydrophiles, reliés en un peptide et qui définit un polypeptide qui relie l'extrémité C-terminale dudit site de liaison et l'extrémité N-terminale dudit premier domaine biofonctionnel ou l'extrémité N-terminale dudit site de liaison et l'extrémité C-terminale dudit premier domaine biofonctionnel, après quoi ladite protéine de liaison adopte une conformation appropriée pour la liaison et ledit premier domaine biofonctionnel adopte une conformation appropriée pour une activité biologique, la séquestration d'un ion ou la liaison sélective à un support solide.
2. La protéine de la revendication 1, dans laquelle ledit site de liaison comprend au moins deux domaines de liaison reliés en un peptide par un second polypeptide de jonction disposé entre lesdits domaines, où ledit second polypeptide de jonction comprend des acides aminés multiples, hydrophiles, reliés en un peptide et qui définit un polypeptide d'une longueur suffisante pour franchir la distance s'éparant l'extrémité C-terminale de l'un desdits domaines de liaison de l'extrémité N-terminale de l'autre desdits domaines de liaison, quand ladite protéine de liaison adopte une conformation appropriée pour la liaison, quand elle est placée en solution aqueuse.
3. La protéine de la revendication 2, dans laquelle la séquence d'acides aminés de chacun desdits domaines de liaison comprend un jeu de CDR interposé entre un jeu de FR, chacun d'entre eux étant respectivement homologue aux CDR et FR de ladite région variable d'une molécule d'immunoglobuline capable de lier ledit déterminant antigénique présélectionné.
4. La protéine de la revendication 2, dans laquelle au moins un desdits domaines de liaison comprend un jeu de CDR en question, homologue aux CDR de la première immunoglobuline et un jeu de FR homologue aux FR d'une seconde immunoglobuline distincte.
5. La protéine de la revendication 1 ou 2, dans laquelle ladite protéine effectrice est une enzyme, une toxine, un récepteur, un site de liaison, un site de liaison d'un anticorps biosynthétique, un facteur de croissance, un facteur de différenciation cellulaire, une lymphokine, une cytokine, une hormone; un fragment détectable à distance ou un anti-métabolite.
6. La protéine de la revendication 1 ou 2, dans laquelle ladite séquence capable de séquestrer un ion est la calmoduline, la métallothionéine, un fragment de celles-ci ou une séquence d'acides aminés riche en au moins l'un d'entre les acide glutamique, acide aspartique, lysine et arginine.
7. La protéine de la revendication 1 ou 2, dans laquelle ladite séquence polypeptidique capable de se lier sélectivement à un support solide est une séquence d'acides aminés chargée positivement ou négativement, une séquence d'acides aminés contenant de la cystéine, la streptavidine ou un fragment de la protéine A de Staphylococcus.

8. La protéine de la revendication 2 comprenant une pluralité de sites de liaison d'un anticorps biosynthétiques, chaque site de liaison comprenant deux desdits domaines de liaison reliés par un polypeptide de jonction.
- 5 9. Un ADN codant pour la protéine de la revendication 1.
10. Une cellule hôte renfermant et étant capable d'exprimer l'ADN de la revendication 9.
11. Une chaîne polypeptidique unique comprenant:
  - 10 une paire de domaines polypeptidiques définissant ensemble un site pour la liaison à un antigène présélectionné et étant joints par l'intermédiaire du C-terminal de l'un et du N-terminal de l'autre, par un polypeptide de jonction, où la séquence d'acides aminés de chacun desdits domaines polypeptidiques mime une région variable d'une immunoglobuline et au moins un desdits domaines comprend:
    - un jeu de séquences d'acides aminés CDR définissant ensemble un site de reconnaissance pour
    - 15 ledit antigène présélectionné, dans lequel lesdites séquences CDR sont des séquences non-humaines,
    - un jeu de séquences d'acides aminés FR reliées audit jeu de séquences CDR, dans lequel lesdites séquences d'acides aminés FR sont homologues aux séquences obtenues à partir d'une immunoglobuline humaine, et
    - lesdits jeux reliés de séquences d'acides aminés CDR et FR définissant ensemble un domaine de
    - 20 liaison chimérique qui, quand il est placé en solution aqueuse, adopte une structure tertiaire appropriée pour une liaison immunologique avec ledit antigène présélectionné.
12. Une chaîne polypeptidique unique comprenant:
  - 25 une paire de domaines polypeptidiques définissant un site pour la liaison à un antigène présélectionné, reliés par un polypeptide de jonction franchissant la distance séparant le C-terminal de l'un du N-terminal de l'autre, dans laquelle la séquence d'acides aminés de au moins un desdits domaines polypeptidiques comprend une région variable recombinante comprenant:
    - un jeu de séquences d'acides aminés CDR définissant ensemble un site de reconnaissance pour
    - 30 ledit antigène présélectionné, dans lequel lesdites séquences CDR sont homologues aux séquences obtenues à partir d'une première immunoglobuline,
    - un jeu de séquences d'acides aminés FR, relié audit jeu de séquences CDR, dans lequel lesdites séquences d'acides aminés FR sont homologues aux séquences obtenues à partir d'une seconde immunoglobuline, et
    - 35 lesdits jeux reliés de séquences d'acides aminés CDR et FR définissent ensemble un polypeptide de liaison d'une région variable, à chaîne unique, chimérique, qui, quand il est placé en solution aqueuse, adopte une structure tertiaire appropriée pour une liaison immunologique avec ledit antigène présélectionné.
13. La chaîne polypeptidique de la revendication 12, dans laquelle les séquences FR sont homologues aux  
40 séquences obtenues à partir d'une immunoglobuline humaine et les séquences CDR sont homologues aux séquences obtenues à partir d'une immunoglobuline murine.
14. La chaîne polypeptidique de la revendication 11 ou 12, dans laquelle ladite paire de domaines polypeptidiques est un peptide relié à un domaine biologiquement actif, par l'intermédiaire d'un second  
45 polypeptide de jonction disposé entre ledit polypeptide de liaison et ledit domaine biologiquement actif, dans laquelle ledit second polypeptide de jonction comprend des acides aminés multiples, hydrophiles, reliés en un peptide, qui, ensemble, adoptent une configuration polypeptidique non-structurée, en solution aqueuse et relient l'extrémité C-terminale dudit site de liaison et l'extrémité N-terminale dudit  
50 domaine biologiquement actif ou l'extrémité N-terminale dudit site de liaison et l'extrémité C-terminale dudit domaine biologiquement actif, après quoi ledit polypeptide de liaison adopte une conformation appropriée pour la liaison et ledit domaine biologiquement actif adopte une conformation appropriée pour une activité biologique, la séquestration d'un ion ou la liaison sélective à un support solide.

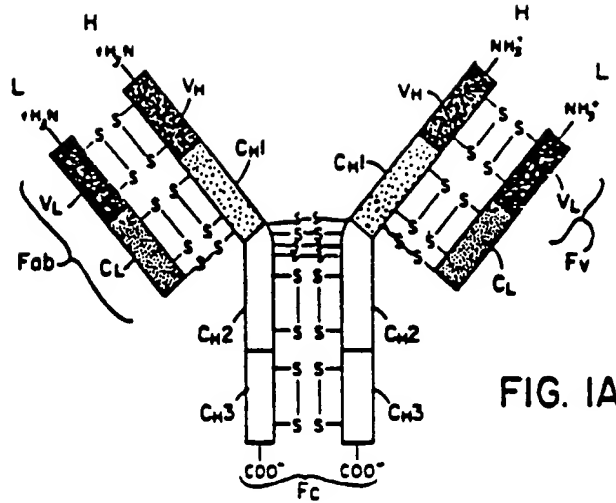
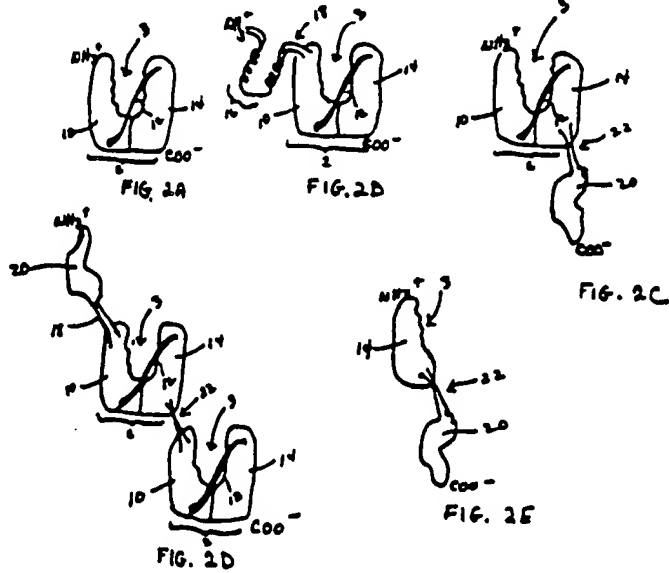


FIG. 1A

CDR1										26-10 GENES/ PROTEINS	
V <sub>H</sub>											
FR1		FR2	CDR2		FR3	CDR3	FR4				
31	35	50	64			100	106	119	AMINO ACID SEQUENCE NO.		
93	115	150	192			300	318	357	DNA BASE NO		
CDR2											
CDR3											
FR1	CDR1	FR2			FR3		FR4				
24	37	55	61			90	101	113	AMINO ACID SEQUENCE NO.		
72	111	165	183			288	303	339	DNA BASE NO.		

FIG. 1B



9-loop:  
 QVQLQSGPELVEPGASVRI SCTASGYTFTNYYIHMLKQRPQCGLIEWIGWYIPGNGHTKYNENFKGKATLTADKSSSTAFNQISSLTSEDSAVYPCARYTHYYP DYWGQGTTLTVSSK\*

26-10:  
 EVQLQSGPELVKPGASVVRMSCKSSGYIFTDFYHNMVVRQSHGKSLDYIGYISPYSGVTGYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCAGSSGNKWAHDYMGHGASVTVSS\*

26-10/g-loop hybrid:  
 EVQLQSGPELVKPGASVVRMSCKSSGYFTFTNYYIHMLKQSHGKSLDWIGWYIPGNGHTKYNENFKGKATLTADKSSSTAYMELRSLTSECSAVYYCAFYTHYF DYMGHGASVTVSS\*  
 (cdr1-----) (cdr2-----) (cdr3-----) nheI  
 hphI bstXI xbaI draI hincII sacII  
 neuM/g-loop hybrid:  
 EVQLQSGPELVKPGASVVRMSCKSSGYFTFTNYYIHMLKQPPGRCGLDWIGWYIPGNGHTKYNENFKGRVTHLVDTSKNQFSRLRLSSVTAADTAIVYYCAFYTHYF DYWGQGS LVTVSS\*  
 (neuM1.....) (neuM2..) (neuM3.....) (neuM4)  
 avaiI bstXI...xbaI draI.....sacII .....

neuM:  
 EVQLQSGPELVKPGASVVRMSCKSSGYFTFTNYYIHMLKQPPGRCGLDWIGWYIPGNGHTKYNENFKGRVTHLVDTSKNQFSRLRLSSVTAADTAIVYYCARHLAGCIDVWGQGS LVTVSS\*

FIG. 3

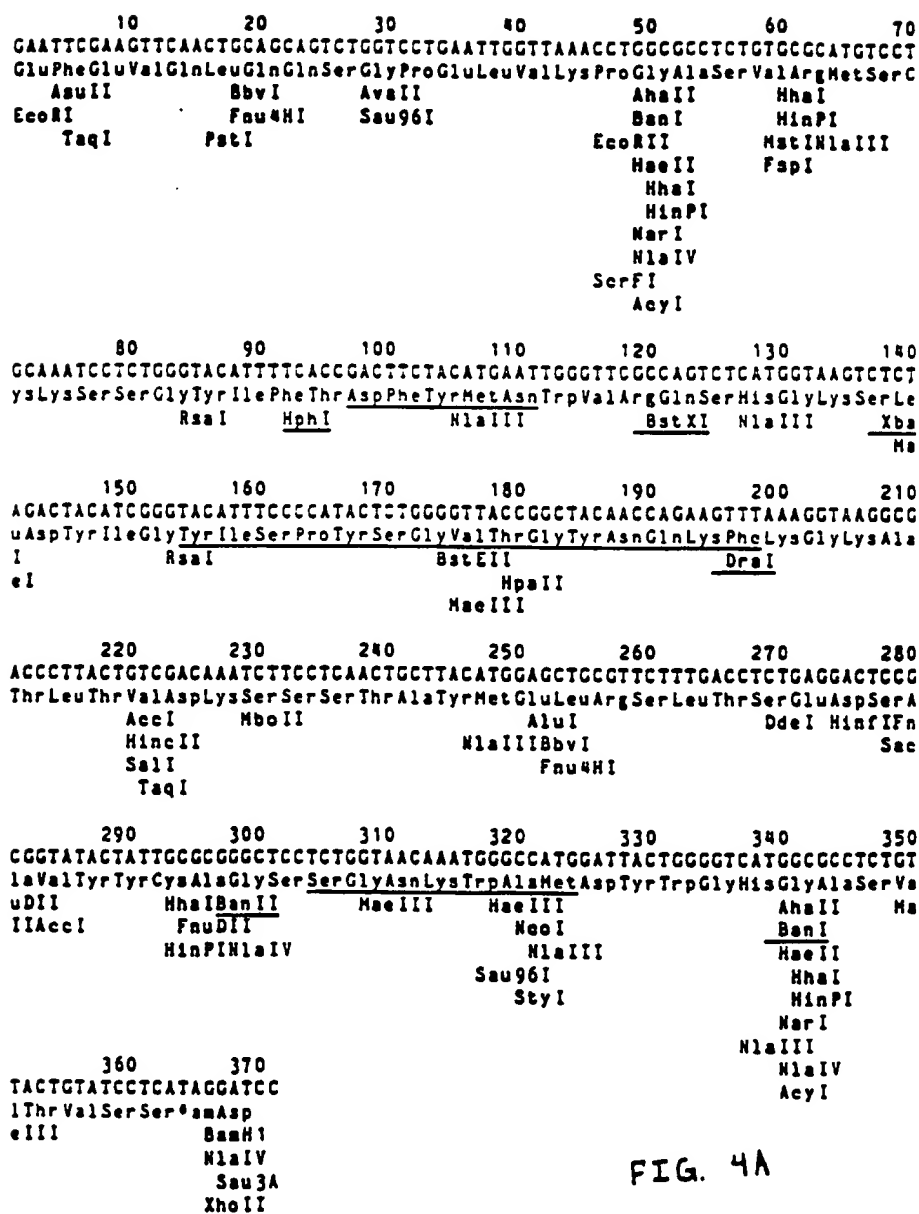


FIG. 4A

10 20 30 40 50 60 70  
 GAATTCGACGTCCTAATGACCCAGACTCCGCTGCTCTGCGCGTTTCTCTGGGTGACCAGGCTTCTATTT  
 GluPheAspValValMetThrGlnThrProLeuSerLeuProValSerLeuGlyAspGlnAlaSerIleS  
 EcoRI AatII HinfI RpaII BstEII  
 AhaII HphI EcoRII  
 TaqI ScrFI  
 AclI MaeIII  
 MaeII

80 90 100 110 120 130 140  
 CTTGCCGCTCTTCCCACTCTCTGGTCCATTCTAATGGTAACACTTACCTGAACTGGTACCTGCAAAAGGC  
 erCysArgSerSerGlnSerLeuValHisSerAsnGlyAsnThrTyrLeuAsnTrpTyrLeuGlnLysAl  
 FnuuHI AatII MaeIII HgiEII BstXI  
 MboII BstXI KpnI  
 Sau96I NlaIV  
 RsaI

150 160 170 180 190 200 210  
 TGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCCTTCTCTGGTGTCCCGGATCGTTTCTCT  
 aGlyGlnSerProLysLeuLeuIleTyrLysValSerAsnArgPheSerGlyValProAspArgPheSer  
 AluI Sau3A HpaII  
 HindIII NciISau3A  
 ScrFI

220 230 240 250 260 270 280  
 CGTTCTCGTTCTGGTACTGACTTCACCCCTGAAGATCTCTCGTCTCGAGGCCGAGGATCTGGGTATCTACT  
 GlySerGlySerGlyThrAspPheThrLeuLysIleSerArgValGluAlaGluAspLeuGlyIleTyrP  
 RsaI HphI BglII TaqIHaeIII Sau3A  
 MboII XhoII  
 Sau3A  
 XhoII

290 300 310 320 330 340 350  
 TCTGCTCTCAGACTACTCATGTACCCCGACCTTCGGCGGTGGCACCAAGCTCGAGATCAAACGTTGAGGATCC  
 heCysSerGlnThrThrHisValProProThrPheGlyGlyGlyThrLysLeuGluIleLysArg\*op  
 DdeI NlaIII HgiEII BstXI AluI Sau3A MaeII BamHI  
 RsaI NlaIV AclI NlaIV  
 TaqI Sau3A  
 XhoI XhoII

FIG. 4B

10 20 30 40 50 60 70  
 GAATTGGAAGTTCAACTGCAGCAGTCTGCTGCTGAATTGGTTAAACCTGGCGGCTCTGTGGCCATGTCCT  
 GluPheGluValGlnLeuGlnGlnSerGlyProGluLeuValLysProGlyAlaSerValArgMetSerC  
 AsuII BbvI AfaII AhaII HhaI  
 EcoRI Fnu4HI Sau96I BstI HinfI  
 TaqI PstI EcoRII HaeII FspI  
 HhaI  
 HinfI  
 NarI  
 NlaIV  
 AclI  
 80 90 100 110 120 130 140  
 GCAATCCTCTCGGTACATTTTACCAATTACTACATCCATTGGGTTGGCCAGTCTCATGGTAAGTCTCT  
 CATGTAAAAGTGGTTAATCATGTACGTAACCCAGCGGTC  
 ysLysSerSerGlyTyrIlePheThrAsnTyrTyrIleHisTrpValArgGlnSerHisGlyLysSerLe  
 RsaI HdeI FokI BstXI NlaIII Xba  
 Ma  
 150 160 170 180 190 200 210  
 AGACTACATCGGGTGGATCTACCCGGGTAATGGTAACACTAAGTACTACAATGAGAACTTTAAAGGTAAG  
 TGATGCTCTCCACCTAGATGGGCGCATACCATTTGTCATTTCATGATGTTACTCTTGA  
 uAspTyrIleGlyTrpIleTyrProGlyAsnGlyAsnThrLysTyrTyrAsnGluAsnPheLysGlyLys  
 I Sau3A AfaI MaeII DdeIRsaI DraI  
 eI XhoII HpaII ScaI  
 NciI  
 NcoI  
 SmaI  
 XbaI  
 220 230 240 250 260 270 280  
 GCGACCCCTTACTGTCGACAAATCTTCCTCAACTGCTTACATGGAGCTGCGTTCTTTGACCTCTGAGGACT  
 AlaThrLeuThrValAspLysSerSerSerThrAlaTyrMetGluLeuArgSerLeuThrSerGluAspS  
 AccI MboII AluI DdeI Hinf  
 HincII NlaIII BbvI  
 SalI Fnu4HI  
 TaqI  
 290 300 310 320 330 340 350  
 CCGCGGTATACTATTGCGCGGGCTCCTCTGCTAACAAATGGGCTTCGATTACTGGGGTCATGGCGGCTC  
 GGAAGCTAATGACCCAGTACCGC  
 erAlaValTyrTyrCysAlaGlySerSerGlyAsnLysTrpAlaPheAspTyrTrpGlyHisGlyAlaSe  
 I AccI HhaIBanII MaeIII HaeIII AhaII  
 FnuDII FnuDII Sau96ITaqI BstI  
 SacII HinfINlaIV HaeII  
 HhaI  
 HinfI  
 NarI  
 NlaIII  
 NlaIV  
 AclI  
 360 370  
 TGTACTGTATCCTCATAGGATCC  
 rValThrValSerSer\*am  
 MaeIII BamHI  
 NlaIV  
 Sau3A  
 XhoII

FIG. 4C



10' 20 30 40 50 60 70  
 GAATTCGACGTCGTAATGACCCAGACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTT  
 GluPheAspValValMetThrGlnThrProLeuSerLeuProValSerLeuGlyAspGlnAlaSerIleS  
 EcoRI AatII HinfI HpaII BstEII  
 AhaII HphI EcoRII  
 TaqI ScrFI  
 AclI MaeIII  
 MaeII  
 80 90 100 110 120 130 140  
 CTTGCCGCTCTTCCCAGTCTATTGTGCACTCTAATGGTAACACTTACCTGGATTGGTACCTGCAAAAGGC  
 AACCGCCGACAAGGGTCAGATAACACGTCAGATTACCATTTGTAATGGACCTAAC  
 erCysArgSerSerGlnSerIleValHisSerAsnGlyAsnThrTyrLeuAspTrpTyrLeuGlnLysAl  
 Fnu4HI HgiAI MaeIII EcoRII BstEII  
 MboII ScrFI KpnI  
 HgiEII NlaIV  
 RsaI  
 150 160 170 180 190 200 210  
 TGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCGCTTCTCTGGTCTCCCGGATCGTTTCTCT  
 aGlyGlnSerProLysLeuLeuIleTyrLysValSerAsnArgPheSerGlyValProAspArgPheSer  
 AluI Sau3A HpaII  
 HindIII NciISau3A  
 ScrFI  
 220 230 240 250 260 270 280  
 GGTCTCTGTTCTGGTACTGACTTCACCCCTGAAGATCTCTCGTGTCCAGGCCGAGGATCTGGGTATCTACT  
 GGTCTCTACACCCATAGATGA  
 GlySerGlySerGlyThrAspPheThrLeuLysIleSerArgValGluAlaGluAspLeuGlyIleTyrT  
 RsaI HphI BglII TaqIHaeIII Sau3A  
 MboII XhoII  
 Sau3A  
 XhoII  
 290 300 310 320 330 340 350  
 ACTGCTTCCAGGGGTCTCATGTACCGTGGACCTTCGGCGGTGGCACCAGCTCGAGATCAAACGTTGAGGATCC  
 TCACCAAGGTCCCCAGAGTACATGGCACCTGGAAGCCGCCACCGTGGTTCCGAGCT  
 yrCysPheGlnGlySerHisValProTrpThrPheGlyGlyGlyThrLysLeuGluIleLysArg\*op  
 EcoRII NlaIII AclI BstEII AluI Sau3A MaeII BamHI  
 ScrFI RsaI Sau96I NlaIV AclI NlaIV  
 HgiEII TaqI XhoI XhoII  
 XhoII

FIG. 4D

10 20 30 40 50 60 70  
 GAATTCATGGAAGTACAACCTGCAACAATCTGGGCCCCGTCTGGTACGTCCTCTCAGACTCTGTCCCTCA  
 GluPheMetGluValGlnLeuGlnGlnSerGlyProGlyLeuValArgProSerGlnThrLeuSerLeuT  
 EcoRI NlaIII RsaI ApeI HpaII RsaI DdeI HinfI  
 BanII MaeII TthIII  
 HaeIII  
 MclI  
 NlaIV  
 Sau96I  
 Sau96I  
 ScrFI  
 80 90 100 110 120 130 140  
 CTTGTACCGTATCCGGATCCACCTTCTCTAACTACTACATCCATTGGGTCCGTCAACCGCCGGTCTGGTGG  
 hrCysThrValSerGlySerThrPheSerAsnTyrTyrIleHisTrpValArgGlnProProGlyArgG1  
 RsaI BamHI FokI AvaII HincII HpaII  
 HpaII NlaIV NciI  
 NlaIV Sau96I ScrFI  
 Sau3A  
 XhoII  
 150 160 170 180 190 200 210  
 TCTCGAGTGGATCGGTTGGATTACCCCGGTAATGGTAACACTAAGTACTACAATCAGAACTTTAAAGGC  
 yLeuGluTrpIleGlyTrpIleTyrProGlyAsnGlyAsnThrLysTyrTyrAsnGluAsnPheLysGly  
 Aval Sau3A Aval MaeII DdeI RsaI DraI N  
 TaqI HpaII SmaI Sp  
 XhoI NciI  
 NciI  
 ScrFI  
 ScrFI  
 SmaI  
 XmaI  
 220 230 240 250 260 270 280  
 ATGCTGGTGGACACTTCTAAGAACCAATTCTCTCTGCGTCTGTCTTCTGTTACCGCGGCTGATACTGCTG  
 MetLeuValAspThrSerLysAsnGlnPheSerLeuArgLeuSerSerValThrAlaAlaAspThrAlaV  
 laIII AccI DdeI XmnI HgaI MboII MaeIII Fnu4HI  
 hI HincII BbvII FnuDII  
 SalI SacII  
 TaqI  
 290 300 310 320 330 340 350  
 TGTACTACTGCGCGCGTTCCTCCGGTAATAAGTGGGCATTGATTACTGGGGCCAGGGCTCTCTGGTCAC  
 alTyrTyrCysAlaArgSerSerGlyAsnLysTrpAlaPheAspTyrTrpGlyGlnGlySerLeuValTh  
 RsaI BssHII HpaII NlaIV BanII BstEII  
 FnuDII EcoRII HphI  
 FnuDII HaeIII MaeIII  
 HhaI Sau96I  
 HhaI ScrFI  
 HinfI  
 HinfI  
 360 370  
 CGTATCTCTTAACTGCAG  
 rValSerSer<sup>o</sup>cLeuGln  
 PstI

FIG. 4E

10 20 30 40 50 60 70  
 GAATTCATGGAATCTGTCTGACTCAGCCGCCGTCTGTATCTGGTGCACCGGGTCAACGGCTAACTATCT  
 GluPheMetGluSerValLeuThrGlnProProSerValSerGlyAlaProGlyGlnArgValThrIleS  
 EcoRI HinfI DdeIFnu4HI HgiAIIHpaII FnuDII  
 NlaIII HinfI NotIIHincII MaeIII  
 XmnI ScrFI MluI  
 80 90 100 110 120 130 140  
 CTGGCCGTTCCTCTCAGTCTATTGTCCATTCTAATGGCAACACTTATCTGGAATGGTACCAACAACCTGCC  
 erCysArgSerSerGlnSerIleValHisSerAsnGlyAsnThrTyrLeuGluTrpTyrGlnGlnLeuPr  
 DdeI BstXI BanI Hp  
 KpnI Ne  
 NlaIV Sc  
 RsaI  
 150 160 170 180 190 200 210  
 GGGCACCGCGCCGAAGCTGCTGATCTTTAAAGTATCTAATCGCTTCTCTGGCGTACCGGATCGATTCTCT  
 oGlyThrAlaProLysLeuLeuIlePheLysValSerAsnArgPheSerGlyValProAspArgPheSer  
 aII FnuDII AluI DraI RsaI ClaI  
 il HhaI BbvI Sau3A HpaII HinfI  
 rFI HinfI Fnu4HI Sau3A  
 BanI  
 NlaIV TaqI  
 220 230 240 250 260 270 280  
 GTATCTAAGTCTGGCTCCTCTGCCACTCTGGCGATCACTGGTCTGCAAGCAGAAGATGAGGCCGATTACT  
 ValSerLysSerGlySerSerAlaThrLeuAlaIleThrGlyLeuGlnAlaGluAspGluAlaAspTyrI  
 DdeI NlaIV BglI Sau3A MboII MaeIII  
 290 300 310 320 330 340 350  
 ACTGTTTTCAAGGCTCTCATGTACCGTGGACCTTCGGTGGTGGCACCAAGCTTACTGTACTGCGTCAGCC  
 yrCysPheGlnGlySerHisValProTrpThrPheGlyGlyGlyThrLysLeuThrValLeuArgGlnPr  
 NlaIII AclI BanI AluI RsaI HgaI  
 RsaI Sau96I NlaIV HindIII  
 HgiEII  
 360  
 GTAACCTGCAG  
 o\*ocLeuGln  
 PstI  
 MaeIII

FIG. 4F

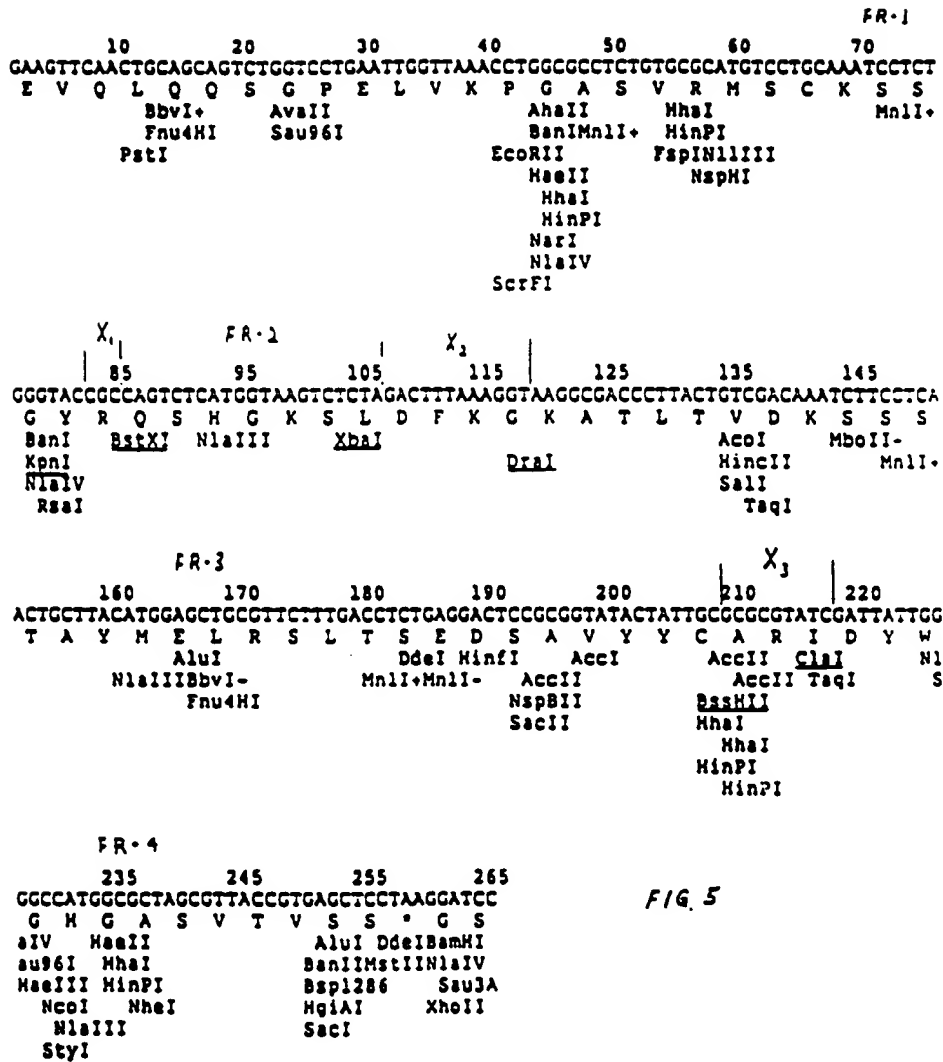


FIG. 5

10 20 30 40 50 60 70  
 GAATTCATGGCTGACAACTTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTGACCTGCCGAACTG  
 E F M A D N K F N K E Q Q N A F Y E I L H L P N L  
 EcoRI MluI BglII BspMI+  
 XbaI

85 95 105 115 125 135 145  
 AACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGACGACCGTCTCAGAGCGCTAACCTGCTGGCAGAG  
 N E E Q R N G F I Q S L K D D P S Q S A N L L A E  
 HindIII BspMI+  
 Eco47III

160 170 180 190 200 210 220  
 GCCAAGAACTGAACGAGCTCAGGCGCCGAAGAGTGATCCCGAAGITCAACTGCAGCAGTCTGGTCTGAATTG  
 A K K L N D A Q A P K S D P E V Q L Q Q S G P E L  
 NarI PstI

235 245 255 265 275 285 295  
 GTTAAACCTGGCGCCTCTGTGCGCATGTCCTGCAAATCCTCTGGGTACATTTTCACCGACTTCTACATGAATTG  
 V K P G A S V R M S C K S S G Y I F T D F Y M N W  
 NarI FspI

310 320 330 340 350 360 370  
 GTTCGCCAGTCTCATGGTAAGTCTCTAGACTACATCGGGTACATTTCCCCATACTCTGGGGTTACCGGCTACAAC  
 V R Q S H G K S L D Y I G Y I S P Y S G V T G Y N  
 BstXI XbaI PflMI BstEII

385 395 405 415 425 435 445  
 CAGAAGTTAAAGGTAAGGCGACCTTACTGTGACAAATCTTCTCAACTGCTTACATGGAGCTGCGTTCTTTG  
 Q K F K G K A T L T V D K S S S T A Y M E L R S L  
 DraI SalI

460 470 480 490 500 510 520  
 ACCTCTGAGGACTCCGCGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCATGGATTATTGGGGTCAT  
 T S E D S A V Y Y C A G S S G N K W A M D Y W G H  
 SacII NcoI

535 545 555 565 575 585 595  
 GGTGCTAGCGTACTGTGAGCTCTGGTGGCGGTGGGTGGCGGTGGTGGCTCGGGTGGCGGCGGATCCGACGTC  
 G A S V T V S S G G G G S G G G G S G G G G S D V  
 NheI SacI BamHI AatII

610 620 630 640 650 660 670  
 GTTGTACCCAGACTCCGCTGTCTCTGCGGTTTCTCTGGGTGACCAGGCTTCTATTCTTGGCGGCTCTTCCAG  
 V V T Q T P L S L P V S L G D Q A S I S C R S S Q  
 BstEII PflMI

685 695 705 715 725 735 745  
 TCTCTGGTCCATTCTAATGGTAACACTTACCTGAACTGGTACCTGCAAAAGGCTGGTCAGTCTCCGAAGCTTCTG  
 S L V H S N G N T Y L N W Y L Q K A G Q S P K L L  
 I BstXI BspMI+ HindIII  
 XpnI

FIG. 6A-1

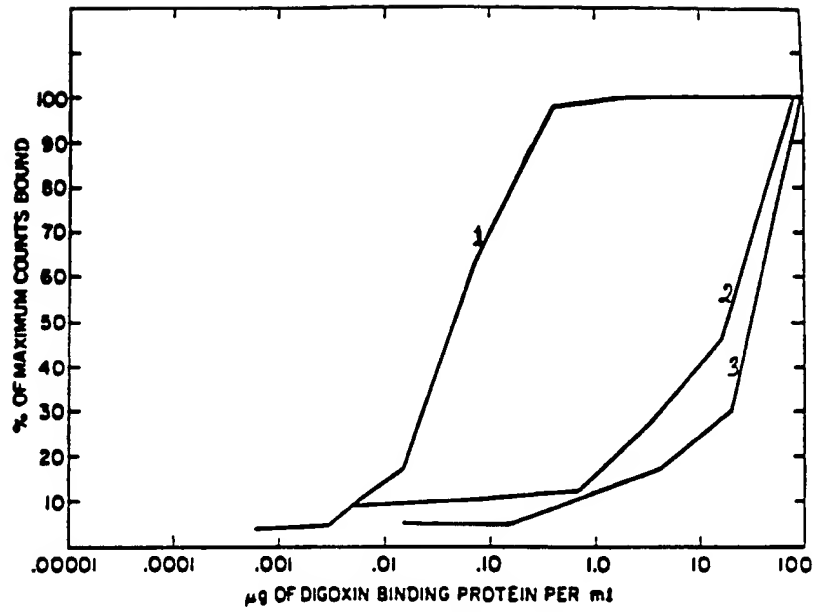


FIG. 7A

```

      760      770      780      790      800      810      820
ATCTACAAAGTCTCTAACCGCTTCTCTGGTGTCCCGGATCGTTTCTCTGGTTCTGGTTCTGGTACTGACTTCACC
I Y K V S N R F S G V P D R F S G S G S G T D F T

      835      845      855      865      875      885      895
CTGAAGATCTCTCGTGTGAGGCCGAAGACCTGGGTATCTACTTCTGCTCTCAGACTACTCATGTACCGCCGACT
L K I S R V E A E D L G I Y F C S Q T T H V P P T
BgIII

      910      920      930      940
TTTGGTGGTGGCACCAAGCTCGAGATTAAACGTAACTGCAG
F G G G T K L E I K R *
      XhoI      HpaI PstI

```

FIG. 6A-2

10 20 30 40 50 60  
 GATCCTGACGTCGTAATGACCCAGACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAG  
 D P D V V M T Q T P L S L P V S L G D Q  
 AatII BstEII

70 80 90 100 110 120  
 GCTTCTATTTCTTGGCGCTCTTCCAGTCTCTGGTCCATTCTAATGGTAACACTTACCTG  
 A S I S C R S S Q S L V H S N G N T Y L  
 PflMI BstXI

130 140 150 160 170 180  
 AACTGGTACCTGCAAAAGGCTGGTCAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAAC  
 N W Y L Q K A G Q S P K L L I Y K V S N  
 BspMI+ HindIII  
 KpnI

190 200 210 220 230 240  
 CGCTTCTCTGGTGTCCCGGATCGTTTCTCTGGTTCTGGTTCTGGTACTGACTTCACCTG  
 R F S G V P D R F S G S G S G T D F T L

250 260 270 280 290 300  
 AAGATCTCTCGTGTGAGGCCGAGACCTGGGTATCTACTTCTGCTCTCAGACTACTCAT  
 K I S R V E A E D L G I Y F C S Q T T H  
 BglII

310 320 330 340 350 360  
 GTACCGCCGACTTTTGGTGGTGGCACCAAGCTCGAGATTAAACGTGGATCTGGAGGTGGC  
 V P F T F G G G T K L E I K R G S G G G  
 XhoI

370 380 390 400 410 420  
 GGATCTGTGGAGGTGGCTCTGGTGGCGGTGGATCCGAAGTTCAATTGCAGCAGTCTGGT  
 G S G G G G S G G G S E V Q L Q Q S G  
 BamHI

430 440 450 460 470 480  
 CCTGAATGGTTAAACCTGGCGCCTCTGTGCGCATGTCTGCAAATCCTCTGGGTACATT  
 P E L V K P G A S V R M S C K S S G Y I  
 NarI FspI

490 500 510 520 530 540  
 TTCACCGACTTCTACATGAATTGGGTTGCCAGTCTCATGGTAAGTCTCTAGACTACATC  
 F T D F Y M N W V R Q S H G K S L D Y I  
 BstXI XbaI

550 560 570 580 590 600  
 GGGTACATTTCCCATACTCTGGGGTTACCGGCTACAACCAGAAGTTTAAAGGTAAGGCG  
 G Y I S P Y S G V T G Y N Q K F K G K A  
 PflMI BstEII DraI

610 620 630 640 650 660  
 ACCCTTACTGTGACAAATCTTCTCAACTGCTTACATGGAGCTGCGTTCTTTGACCTCT  
 T L T V D K S S S T A Y M E L R S L T S  
 SalI

670 680 690 700 710 720  
 GAGGACTCCGCGGTATACTATTGCGCGGGCTCCTCTGGTAACAAATGGGCCATGGATTAT  
 E D S A V Y Y C A G S S G N K W A M D Y  
 SacII NcoI

730 740 750 760  
 TGGGGTCATGGTCTAGCGTTACTGTGASCTCTTAACTGCAG  
 W G H G A S V T V S S

FIG. 6B

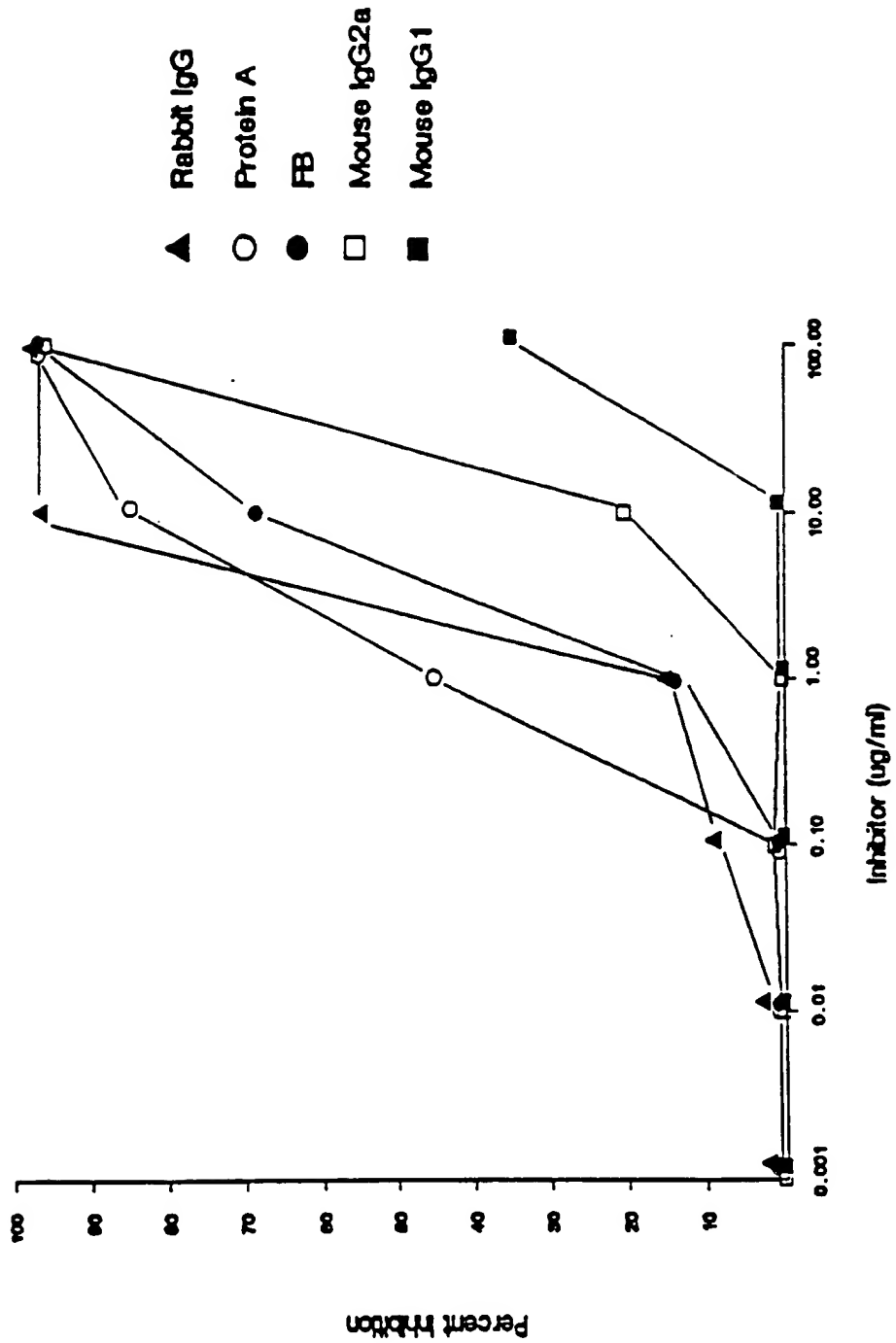


FIG. 7B



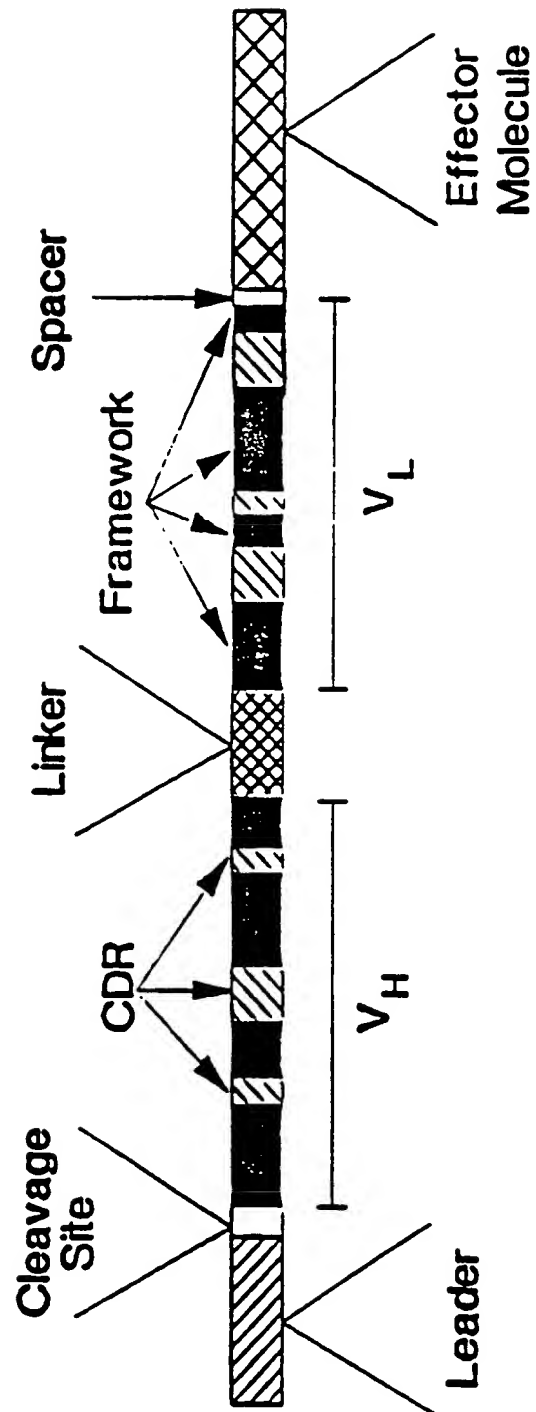


FIG. 8

10 20 30 40 50 60  
 GAAGTTCAACTGGAGCAGTCTGGTCTGGATTGGTTTCGACCTTCCCAGACTCTGTCCCTG  
 E V Q L E Q S G P G L V R P S Q T L S L  
  
 70 80 90 100 110 120  
 ACCTGCACATCCTCTGGGTACATTTTCACCGACTTCTACATGAATTGGGTTCCGCCAGCCT  
 T C T S S G Y I F T D F Y M N W V R Q P  
 BspMI+ BstXI  
  
 130 140 150 160 170 180  
 CCTGGTCCGGGTCTAGACTACATCGGGTACATTTCCCATACTCTGGGGTTACCGGCTAC  
 P G R G L D Y I G Y I S P Y S G V T G Y  
 XbaI PflMI BstEII  
  
 190 200 210 220 230 240  
 AACCAGAAGTTTAAAGGTAAGCGGACCCCTTCTGGTCAACAAATCTAAGAACCAGGCTTCC  
 N Q K F K G K A T L L V N K S K N Q A S  
 DraI  
  
 250 260 270 280 290 300  
 CTGCGGCTGTCTTCTGTGACCGCTGCGGACACCGCGGTATACTATTGCGCGGGCTCCTCT  
 L R L S S V T A A D T A V Y Y C A G S S  
 SacII  
  
 310 320 330 340 350 360  
 GGTAACAAATGGGCCATGGATTATTGGGGTCAGGGTTCTCTGGTTACTGTGAGCTCTGGT  
 G N K W A M D Y W G Q G S L V T V S S G  
 NcoI SacI  
  
 370 380 390 400 410 420  
 GCGGGTGGGTCCGGGCGGTGGTGGCTCGGGTGGCGGCGGATCCGACGTCGTTATGACCCAG  
 G G G S G G G S G G G G S D V V M T Q  
 BamHI AatII  
  
 430 440 450 460 470 480  
 CCTCCGTCGGTTTCGGGGGCTCCTGGTCCAGCGGGTTACTATTCTTGCCGCTCTTCCCAG  
 P P S V S G A P G Q R V T I S C R S S Q  
 PflMI  
  
 490 500 510 520 530 540  
 TCTCTGGTCCATTCTAATGGTAACACTTACCTGAAGTGGTACCAGCAACTGCCTGGTACG  
 S L V H S N G N T Y L N W Y Q Q L P G T  
 I BstXI KpnI  
  
 550 560 570 580 590 600  
 GCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCCTTCTCTGGTGTCCCGGATCGTTTC  
 A P K L L I Y K V S N R F S G V P D R F  
 HindIII  
  
 610 620 630 640 650 660  
 TCTGGTTCTGGTTCTGGTACTGACTTCACCCCTGGCGATCACTGGTCTCCAGGCCGAAGAC  
 S G S G S G T D F T L A I T G L Q A E D  
  
 670 680 690 700 710 720  
 GAGGCTGACTACTTCTGCTCTCAGACTACTCATGTACCGCCGACTTTTGGTGGTGGCACC  
 E A D Y F C S Q T T H V P P T F G G G T  
  
 730 740 750  
 AAGCTCACGGTTCTGCGTTAACTGCAG  
 K L T V L R \* L Q  
 HpaI PstI

FIG. 9A

10 20 30 40 50 60  
 GAATTCGAAGTTCAACTGCAGCAGTCTGGTCCTGAATTGGTTAAACCTGGCGCCTCTGTG  
 E F E V Q L Q Q S G P E L V K P G A S V  
 AsuII PstI NarI PstI  
 EcoRI

70 80 90 100 110 120  
 CGCATGTCCTGCAAAATCCTCTGGGTACACCTTCACCAACTATTACATCCACTGGCTTAAG  
 R M S C K S S G Y T F T N Y Y I H W L K  
 PstI AflIII

130 140 150 160 170 180  
 CAGTCTCATGGTAAGTCTCTAGAGTGGATCGGTTGGATTACCCGGGTAATGGTAACACT  
 Q S H G K S L E W I G W I Y P G N G N T  
 XbaI SmaI

190 200 210 220 230 240  
 AAGTACAATGAGAACTTTAAAGGTAAGGCGACCCCTTACTGTGACAAATCTTCTCTCAACT  
 K Y N E N F K G K A T L T V D K S S S T  
 DraI SalI

250 260 270 280 290 300  
 GCTTACATGGAGCTCGCTTCTTTGACCTCTGAGGACTCCGCGGTATACTATTGCGCGCGT  
 A Y M E L R S L T S E D S A V Y Y C A R  
 SacII BsaHII

310 320 330 340 350 360  
 TAACTCAATTATTACTTCGATTATTGGGGCCATGGCGCTAGCGTTACCGTGAGCTCTGGT  
 Y T H Y Y F D Y W G H G A S V T V S S G  
 NcoI NheI SacI

370 380 390 400 410 420  
 GCGCGTGGCTCGGGCGGTGGTGGGTGGGTGGCGCGGATCCGACGTCGTTATGACCCAG  
 G G G S G G G S G G G G S D V V M T Q  
 BamHI AatII

430 440 450 460 470 480  
 ACTCCGCTGTCTCTGCCGGTTTCTCTGGGTGACCAGGCTTCTATTCTTGGCGCTCTTCC  
 T P L S L P V S L G D Q A S I S C R S S  
 BstEII

490 500 510 520 530 540  
 CAGTCTATCGTCCATTCTAATGGTAACACTTACCTGGAGTGGTACCTGCAAAAGGCTGGT  
 Q S I V H S N G N T Y L E W Y L Q K A G  
 BstXI BspMI+ KpnI

550 560 570 580 590 600  
 CAGTCTCCGAAGCTTCTGATCTACAAAGTCTCTAACCCTTCTCTGGTGTCCCGGATCGT  
 Q S P K L L I Y K V S N R F S G V P D R  
 HindIII

610 620 630 640 650 660  
 TTCTCTGGTTCTGGTTCTGGTACTGACTTCACCTGAAGATCTCTCGTGTGAGGCCGAG  
 F S G S G S G T D F T L K I S R V E A E  
 BglIII

670 680 690 700 710 720  
 GATCTGGGTATCTACTACTGCTTCCAAGGCTCTCATGTACCGTGGACTTTCCGGCGGTGGG  
 D L G I Y Y C F Q G S H V P W T F G G G

730 740 750  
 ACCAAGCTCGAGATTAAACGTTAACTGCAG  
 T K L E I K R + L Q  
 XhoI HpaI PstI

FIG. 9B

10 20 30 40 50 60  
 GATCCCGAGGTTATGCTGGTTGAATCTGGTGGAGTACTGATGGAACCTGGTGGGTCCCTG  
 D P E V M L V E S G G V L M E P G G S L  
 ScaI EcoO

70 80 90 100 110 120  
 AAGCTGAGCTGTGCTGCTAGCGGCTTCACGTTCTCTCGTTACGCCATGTCTTGGGTCCGT  
 K L S C A A S G F T F S R Y A M S W V R  
 EspI NheI PflMI

130 140 150 160 170 180  
 CAGACTCCGGAGAAGCGTCTAGAGTGGGTCCGGACGATATCTTCTGGTGGTTCTCACACG  
 Q T P E K R L E W V A T I S S G G S H T  
 BspMII XbaI NruI EcoRV

190 200 210 220 230 240  
 TTCCATCCAGACAGTGTGAAGGGTCGATTACGATCTCTCGAGACAACGCTAAGAACACG  
 F H P D S V K G R F T I S R D N A K N T  
 XhoI

250 260 270 280 290 300  
 TTGTACCTGCAAATGTCTTCTCTACGTAGTGAAGATACTGCTATGTACTACTGTGCACGT  
 L Y L Q M S S L R S E D T A M Y Y C A R  
 BspMI+ SnaBI ApaLI

310 320 330 340 350 360  
 CCTCCACTGATCTCACTAGTTGCTGATTATGCCATGGATTATTGGGGTCATGGTGCTAGC  
 P P L I S L V A D Y A M D Y W G H G A S  
 SpeI NcoI NheI

370 380 390 400 410 420  
 GTTACTGTGAGCTCTGGTGGCGGTGGGTCCGGCGGTGGTGGCTCGGGTGGCGGCGGATCG  
 V T V S S G G G G S G G G G S G G G G S  
 SacI

430 440 450 460 470 480  
 GATATCGTTATGACTCAGTCTCATAAGTTTCATGTCCACTTCTGTTGGTGACCGTGTCTTCT  
 D I V M T Q S H K F M S T S V G D R V S  
 EcoRV BstEII

490 500 510 520 530 540  
 ATCACTTGTAAGGCCAGCCAGGATGTGGGTGCTGCTATCGCATGGTATCAGCAGAAGCCC  
 I T C K A S Q D V G A A I A W Y Q Q K P  
 PflMI Sma

550 560 570 580 590 600  
 GGGCAGTCTCCTAAGCTGCTGATCTACTGGGCGTGGACTCGTCATACTGGTGTCCCGGAT  
 G Q S P K L L I Y W A S T R H T G V P D  
 I SalI

610 620 630 640 650 660  
 CGTTTCACTGGGTCCGGATCAGGTACTGATTTCACCTCTGACTATTTTCGAACGTTCACTCT  
 R F T G S G S G T D F T L T I S N V Q S  
 BspMII AsuII

670 680 690 700 710 720  
 GATGACCTGGCTGATTACTTCTGCCAGCAATATTCCGGGTACCCTCTGACTTTCCGGTGCC  
 D D L A D Y F C Q Q Y S G Y P L T F G A  
 SspI KpnI Nae

730 740 750  
 GGCCTAAACTCGAGCTGAAGTAACTGCAG  
 G T K L E L K \*  
 I Xh I PstI

FIG. 9D

10 20 30 40 50 60  
 GATCCCGAGGTTATGCTGGTTGAATCTGGTGGAGTACTGATGGAACCTGGTGGGTCCCTG  
 D P E V M L V E S G G V L M E P G G S L  
 ScaI EcoO

70 80 90 100 110 120  
 AAGCTGAGCTGTGCTGCTAGCGGCTTCACGTTCTCTCGTTACGCCATGTCTTGGGTCCGT  
 K L S C A A S G F T F S R Y A M S W V R  
 EspI NheI PflMI

130 140 150 160 170 180  
 CAGACTCCGGAGAAGCGTCTAGAGTGGGTCCGCGACGATATCTTCTGGTGGTTCGAACACT  
 Q T P E K R L E W V A T I S S G G S N T  
 BspMII XbaI NruI EcoRV AsuII

190 200 210 220 230 240  
 TACTATCCAGACAGTGTGAAGGGTCGATTACGATCTCTCGAGACAACGCTAAGAACACG  
 Y Y P D S V K G R F T I S R D N A K N T  
 XhoI

250 260 270 280 290 300  
 TTGTACCTGCAAAATGTCTTCTCTACGTAGTGAAGATACTGCTATGTACTACTGTGCACGT  
 L Y L Q M S S L R S E D T A M Y Y C A R  
 BspMI+ SnaBI ApaLI

310 320 330 340 350 360  
 CCTCCACTGATCTCACTAGTTGCTGATTATGCCATGGATTATTGGGGTCATGGTGCTAGC  
 P P L I S L V A D Y A M D Y W G H G A S  
 SpeI NcoI NheI

370 380 390 400 410 420  
 GTTACTGTGAGCTCTGGTGGCGGTGGGTGGGCGGTGGTGGGCTCGGGTGGCGGCGGATCG  
 V T V S S G G G G S G G G G S G G G G S  
 SacI

430 440 450 460 470 480  
 GATATCGTTATGACTCAGTCTCATAAGTTCATGTCCACTTCTGTTGGTGACCGTGTCTTCT  
 D I V M T Q S H K F M S T S V G D R V S  
 EcoRV BstEII

490 500 510 520 530 540  
 ATCACTTGTAAAGGCCAGCCAGGATGTGGGTGCTGCTATCGCATGGTATCAGCAGAAGCCC  
 I T C K A S Q D V G A A I A W Y Q Q K P  
 PflMI Sma

550 560 570 580 590 600  
 GGGCAGTCTCCTAAGCTGCTGATCTACTGGGCGTGGTCTGCTCATACTGGTGTCCCGGAT  
 G Q S P K L L I Y W A S T R H T G V P D  
 I SalI

610 620 630 640 650 660  
 CGTTTCACTGGGTCCGGATCAGGTACTGATTTCACTCTGACTATTTGGAACGTTTCAGTCT  
 R F T G S G S G T D F T L T I S N V Q S  
 BspMII AsuII

670 680 690 700 710 720  
 GATGACCTGGCTGATTACTTCTGCCAGCAATATTCGGGTACCTCTGACTTTCGGTGGC  
 D D L A D Y F C Q Q Y S G Y P L T F G A  
 SspI KpnI Nae

730 740 750  
 GGCCTAACTCGAGCTGAAGTAACTGCAG  
 G T K L E L K \*  
 I XhoI PstI

FIG. 9E

	10		20
Met Lys Ala Ile Phe Val Leu Lys Gly Ser Leu Asp Arg Asp Leu Asp Ser Arg Leu Asp			
ATG AAA GCA ATT TTC GTA CTG AAA GGT TCA CTG GAC AGA GAT CTG GAC TCT CGT CTG GAT			
		BglII	
	30		40
Leu Asp Val Arg Thr Asp His Lys Asp Leu Ser Asp His Leu Val Leu Val Asp Leu Ala			
CTG GAC GTT CGT ACC GAC CAC AAA GAC CTG TCT GAT CAC CTG GTT CTG GTC GAC CTG GCT			
		BclI	SalI
	50		60
Arg Asn Asp Leu Ala Arg Ile Val Thr Pro Gly Ser Arg Tyr Val Ala Asp Leu Glu Phe			
CGT AAC GAC CTG GCT CGT ATC GTT ACT CCC GGG TCT CGT TAC GTT GCG GAT CTG GAA TTC			
		SmaI	EcoRI

Asp  
GAT

FIG. 10A

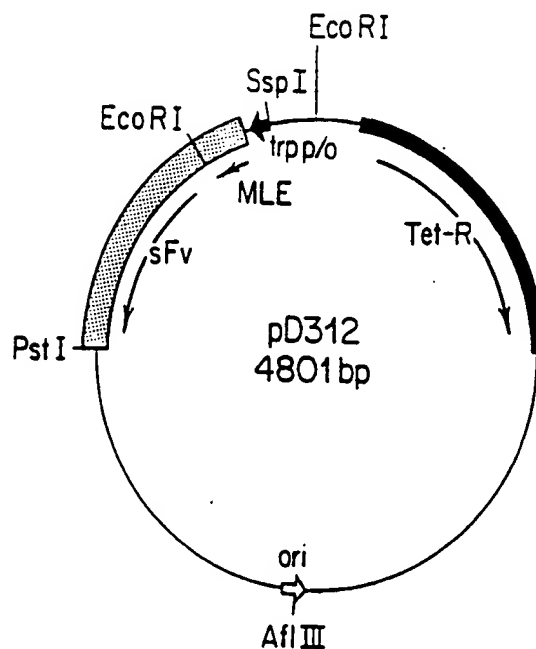


FIG. 10B

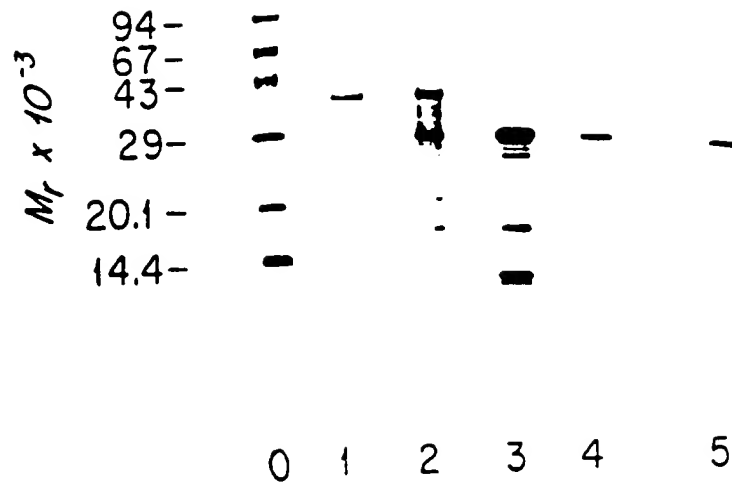


FIG. 11

D V Q L Q E S G P G L V K P S Q S L S L T C S V T G Y S I T  
 S G Y F W N W I R Q F P G N K L E W L G F I K Y D G S N Y G  
 N P S L K N R V S I T R D T S E N Q F F L K L D S V T T A T  
 Y Y C A G D N D H L Y F D Y W G Q G T T L T V S

G G G G S G G G G S G G G G S

Q A V V T Q E S A L T T S P G G T V I L T C R S S T G A V T  
 T S N Y A N W I Q E K P D H L F T G L I G G T S N R A P G V  
 P V R F S G S L I G D K A A L T I T C A Q T E D D A M Y F C  
 A L W F R N H F V F G G G T K V T V L G

FIG. 9C

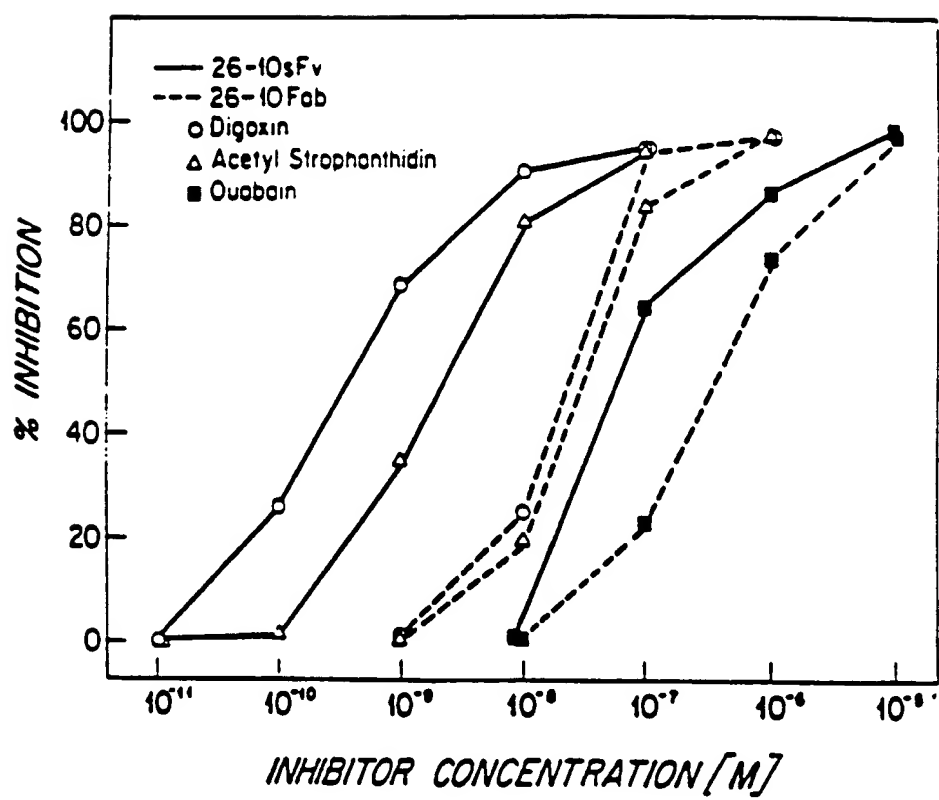
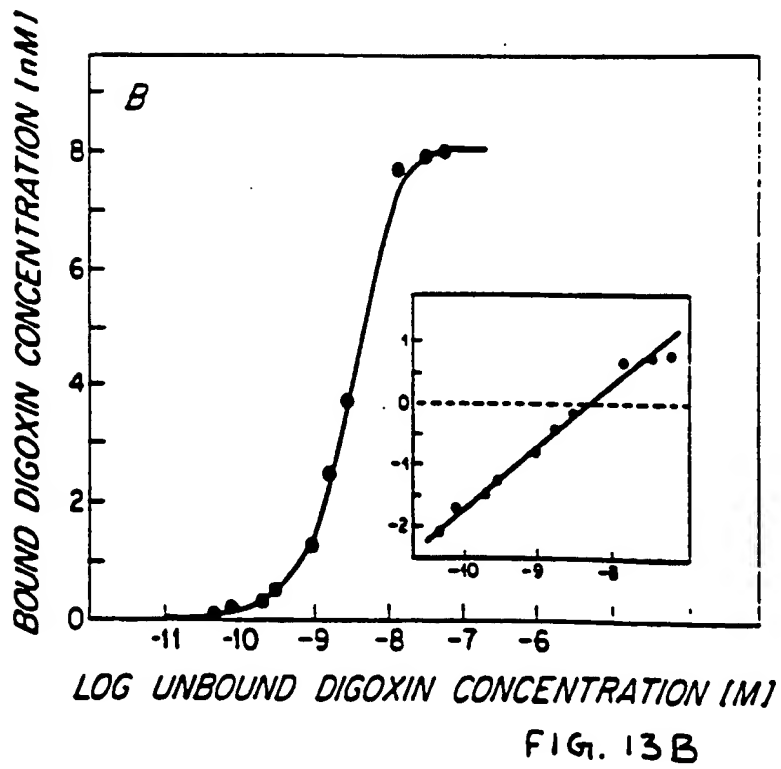
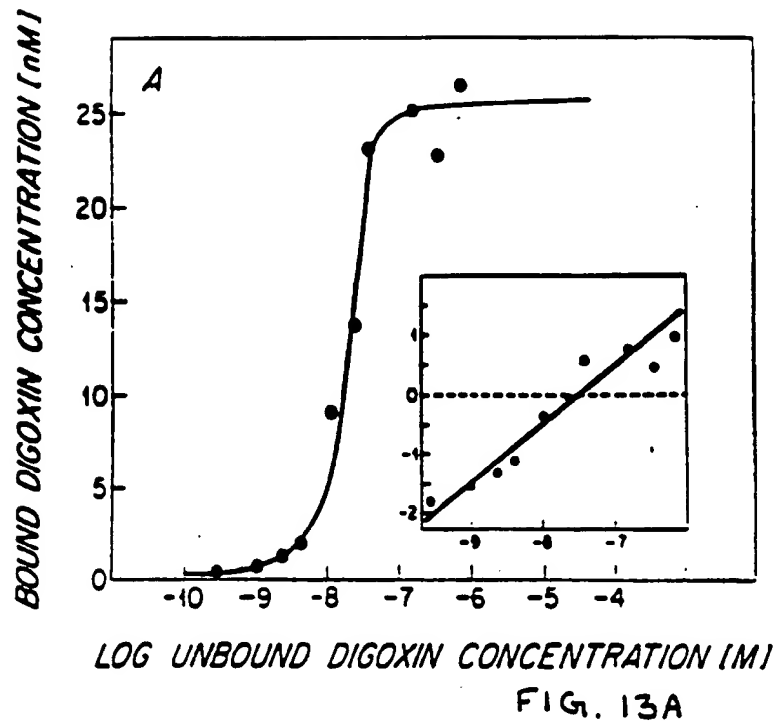


FIG. 12





10 20 30 40 50 60  
 GAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG  
 E F M A D N K F N K E Q Q N A F Y E I L  
 EcoRI MluI BglII  
 XmnI

70 80 90 100 110 120  
 CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG  
 H L P N L N E E Q R N G F I Q S L K D E  
 BspMI+ HindIII

130 140 150 160 170 180  
 CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCG  
 P S Q S A N L L A D A K K L N D A Q A P  
 NheI FspI

190 200 210 220 230 240  
 AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG  
 K S D Q G Q F M A D N K F N K E Q Q N A  
 MluI  
 XmnI

250 260 270 280 290 300  
 TTCTACGAGATCTTGACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA  
 F Y E I L H L P N L N E E Q R N G F I Q  
 BglII BspMI+ H

310 320 330 340 350 360  
 AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAAC  
 S L K D E P S Q S A N L L A D A K K L N  
 indIII NheI

370 380  
 GATGCGCAGGCACCGAAATCGGATCC  
 D A Q A P K S D P  
 FspI BamHI

FIG. 14

(BABS) -

10	20	30	40	50	60	70																		
GGATCCGGTAACTCTGACTCTGAATGCCCGCTGAGCCACGACGGGTACTGCCTGCACGACGGTGTTCATGTAC																								
G	S	G	N	S	D	S	E	C	P	L	S	H	D	G	Y	C	L	H	D	G	V	C	M	Y
BamHI			BsmI+			EspI																		
85	95	105	115	125	135	145																		
ATCGAAGCTCTGGACAAATACGCATGCAACTGCGTTGTAGGCTACATCGGTGAGCGCTGCCAGTATCGCGATCTG																								
I	E	A	L	D	K	Y	A	C	N	C	V	V	G	Y	I	G	E	R	C	Q	Y	R	D	L
						SphI						NruI												
160	170																							
AAATGGTGGGAGCTGCGTTAACTGCAG																								
K	W	W	E	L	R	*																		
					HpaI PstI																			

FIG. 15A

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTGGCGACCCGTCCAAGGACTCCAAAGCTCAGGTTTCTGCTGCCGAAGCTGGT
G S G G D P S K D S K A Q V S A A E A G
BamHI

      70      80      90      100     110     120
ATCACTGGCACCTGGTATAACCAACTGGGGTCGACTTTCATTGTGACCGCTGGTGCGGAC
I T G T W Y N Q L G S T F I V T A G A D
      Sali

     130     140     150     160     170     180
GGAGCTCTGACTGGCACCTACGAATCTGCGGTTGGTAACGCAGAATCCCCTACGTACTG
G A L T G T Y E S A V G N A E S R Y V L
      SacI                               SnaBI

     190     200     210     220     230     240
ACTGGCCGTTATGACTCTGCACCTGCCACCGATGGCTCTGGTACCGCTCTGGGCTGGACT
T G R Y D S A P A T D G S G T A L G W T
      BspMI+                               KpnI

     250     260     270     280     290     300
GTGGCTTGAAAAACAACCTATCGTAATGCGCACAGCGCCACTACGTGGTCTGGCCAATAC
V A W K N N Y R N A H S A T T W S G Q Y
      FspI                               DraIII   Bali
      PflMI                               BstXI

     310     320     330     340     350     360
GTTGGCGGTGCTGAGGCTCGTATCAACACTCAGTGGCTGTAAACATCCGGCACTACCGAA
V G G A E A R I N T Q W L L T S G T T E
      DraIII                               HpaI

     370     380     390     400     410     420
GCGAATGCATGGAAATCGACACTAGTAGGTCATGACACCTTTACCAAAGTTAAGCCTTCT
A N A W K S T L V G H D T F T K V K P S
      BsmI+                               SpeI
      NsiI

     430     440     450     460     470     480
GCTGCTAGCATTGATGCTGCCAAGAAAGCAGGCGTAAACAACGGTAACCCTCTAGACGCT
A A S I D A A K K A G V N N G N P L D A
      NheI                               BstEII   XbaI

     490     500
GTTCAAGCAATAACTGCAG
V Q Q *
      PstI

```

FIG. 15B

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGGTGACGTAGCTCCTCTCGCACTCCGTCGGATAAGCCGGTTGCTCATGTAGTT
  G  S  G  V  R  S  S  S  R  T  P  S  D  K  P  V  A  H  V  V
BamHI      SnaBI

      70      80      90      100      110      120
GCTAACCCTCAGGCAGAAGGTCAGCTTCAGTGGCTGAACCGTCGCGCTAACGCCCTGCTG
  A  N  P  Q  A  E  G  Q  L  Q  W  L  N  R  R  A  N  A  L  L
      MstII                                BglI

      130      140      150      160      170      180
GCAAACGGCGTTGAGCTCCGTGATAACCAGCTCGTGGTACCTTCTGAAGGTCTGTACCTG
  A  N  G  V  E  L  R  D  N  Q  L  V  V  P  S  E  G  L  Y  L
      SacI      PflMI      KpnI

      190      200      210      220      230      240
ATCTATTCTCAAGTACTGTTCAAGGGTCAGGGCTGCCCCGTCGACTCATGTTCTGCTGACT
  I  Y  S  Q  V  L  F  K  G  Q  G  C  P  S  T  H  V  L  L  T
      ScaI                                SalI

      250      260      270      280      290      300
CACACCATCAGCCGTATTGCTGTATCTTACCAGACCAAAGTTAACCTGCTGAGCGCTATC
  H  T  I  S  R  I  A  V  S  Y  Q  T  K  V  N  L  L  S  A  I
      HpaIBspMI+ Eco47III
      EspI

      310      320      330      340      350      360
AAGTCTCCGTGCCAGCGTGAAACTCCCGAGGGTGACAGAAAGCGAAACCATGGTATGAACCG
  K  S  P  C  Q  R  E  T  P  E  G  A  E  A  K  P  W  Y  E  P
      NcoI

      370      380      390      400      410      420
ATCTACCTGGGTGGCGTATTTCAACTGGAGAAAGGTGACCGTCTGTCCGCAGAAATCAAC
  I  Y  L  G  G  V  F  Q  L  E  K  G  D  R  L  S  A  E  I  N
      BstEII

      430      440      450      460      470      480
CGTCCTGACTATCTAGATTTTCGCTGAATCTGGCCAGGTGTACTTCGGTATTATCGCACTG
  R  P  D  Y  L  D  F  A  E  S  G  Q  V  Y  F  G  I  I  A  L
      XbaI      Bali

      490
TAAC TGCAG
*
PstI

```

FIG. 15C

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTGCTGATCAGCTGACTGACGAGCAGATCGCTGAATTTAAAGAGGCTTTCTCT
G S G A D Q L T D E Q I A E F K E A F S
BamHI      BclIPvuII      DraI

      70      80      90      100      110      120
CTGTTTGACAAAGACGGTGACGGTACCATCACTACCAAAGAGCTCGGCACCGTTATGCGC
L F D K D G D G T I T T K E L G T V M R
                KpnI      SacI      FspI

      130      140      150      160      170      180
AGCCTTGGCCAGAACCCGACTGAAGCTGAATTGCAGGACATGATCAACGAAGTCGACGCT
S L G Q N P T E A E L Q D M I N E V D A
      Bali      BclI      SalI

      190      200      210      220      230      240
GACGGTAACGGCACCATCGATTTTCCGGAATTTCTGAACCTGATGGCGCGCAAGATGAAA
D G N G T I D F P E F L N L M A R K M K
                ClaI      BspMII      BssHII

      250      260      270      280      290      300
GACACTGACTCTGAAGAGGAACTGAAAGAGGCCTTCCGTGTTTTCGACAAAGACGGTAAC
D T D S E E E L K E A F R V F D K D G N
                StuI

      310      320      330      340      350      360
GGTTTCATCTCGGCCGCTGAACTGCGTCACGTTATGACTAACCTGGGTGAAAAGCTTACT
G F I S A A E L R H V M T N L G E K L T
      EagI      HindIII

      370      380      390      400      410      420
GACGAAGAAGTTGACGAAATGATTCGCGAAGCTGACGTCGATGGTGACGGCCAGGTTAAC
D E E V D E M I R E A D V D G D G Q V N
                XmnI      NruI      AatII      HpaI

      430      440      450
TACGAAGAGTTCGTTTCAGGTTATGATGGCTAAGTAACTGCAG
Y E E F V Q V M M A K *
                PstI

```

FIG. 15D

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTGGAGGCTCTCTGGGCTCTCTGACTATTGCCGAACCGGCAATGATTGCTGAA
G S G G G S L G S L T I A E P A M I A E
BamHI                               BglI                               Bsm

      70      80      90      100     110     120
TGCAAGACTCGTACCGAAGTCTTCGAGATCTCTCGTCGTCTGATCGATCGCACTAATGCC
C K T R T E V F E I S R R L I D R T N A
I+                               BglII                               ClaI                               Bs
                                      PvuI

      130     140     150     160     170     180
AACTTCCTGGTATGGCCGCGTGCCTCGAGGTACAACGCTGCTCCGGGTGTTGCAACAAT
N F L V W P P C V E V Q R C S G C C N N
tXI

      190     200     210     220     230     240
CGTAACGTTCAATGTGCGACCGACTCAAGTCCAGCTGCGTCCGGTCCAAGTCCGCAAATC
R N V Q C R P T Q V Q L R P V Q V R K I
Sall                               PvuII

      250     260     270     280     290     300
GAGATTGTACGTAAGAAACCGATCTTTAAGAAGGCCACTGTTACTCTGGAAGACCATCTG
E I V R K K P I F K K A T V T L E D H L
SnaBI

      310     320     330     340     350
GCATGCAAATGTGAGACTGTAGCGGCCGCACGTCCAGTTACTTAACTGCAG
A C K C E T V A A A R P V T *
SphI                               EagI                               PstI
                                   NotI

```

FIG. 15E

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTATATTCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCCACT
G S G I F P K Q Y P I I N F T T A G A T
BamHI

      70      80      90      100      110      120
GTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGGTCGTTTAACAACTGGAGCTGAT
V Q S Y T N F I R A V R G R L T T G A D

      130      140      150      160      170      180
GTGAGACATGAAATACCAGTGTGCGCAAACAGAGTTGGTTTGCCTATAAAACCAACGGTTT
V R H E I P V L P N R V G L P I N Q R F

      190      200      210      220      230      240
ATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACC
I L V E L S N H A E L S V T L A L D V T
Eco47III

      250      260      270      280      290      300
AATGCATATGTGGTCCGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACAAT
N A Y V V G Y R A G N S A Y F F H P D N
NdeI
NsiI

      310      320      330      340      350      360
CAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAATCGATATACATTC
Q E D A E A I T H L F T D V Q N R Y T F
ClaI

      370      380      390      400      410      420
GCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATC
A F G G N Y D R L E Q L A G N L R E N I

      430      440      450      460      470      480
GAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGTACTGGT
E L G N G P L E E A I S A L Y Y Y S T G
Eco47III ScaI

      490      500      510      520      530      540
GGCACTCAGCTTCCAACCTCTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTTCAGAA
G T Q L P T L A R S F I I C I Q M I S E

      550      560      570      580      590      600
GCAGCAAGATTCCAATATATGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGA
A A R F Q Y I E G E M R T R I R Y N R R
FspI Bgl

```

FIG. 15F



(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTGCTCCGACTTCTAGCTCTACTAAGAAAACTCAGCTTCAGCTGGAACACCTG
G S G A P T S S S T K K T Q L Q L E H L
BamHI                               PvuII

      70      80      90      100     110     120
CTGCTGGACCTTCAGATGATCCTGAACGGTATCAACAACCTACAAGAACCCGAACTGACT
L L D L Q M I L N G I N N Y K N P K L T

      130     140     150     160     170     180
CGTATGCTGACTTTCAAATTCTACATGCCGAAGAAAGCTACCGAACTGAAACACCTTCAG
R M L T F K F Y M P K K A T E L K H L Q

      190     200     210     220     230     240
TGCCTGGAAGAAGAACTGAAGCCGCTGGAGGAAGTACTGAACCTGGCTCAGTCTAAAAAC
C L E E E L K P L E E V L N L A Q S K N
                               ScaI

      250     260     270     280     290     300
TTCCACCTGCGTCCGCGTGACCTGATCAGCAACATCAACGTAATCGTTCTAGAACTTAAA
F H L R P R D L I S N I N V I V L E L K
                               BclI                               XbaI

      310     320     330     340     350     360
GGCTCTGAAACTACCTTCATGTGCGAATACGCTGACGAAACTGCTACCATCGTAGAATTT
G S E T T F M C E Y A D E T A T I V E F

      370     380     390     400     410     420
CTGAACCGTTGGATCACCTTCTGCCAGTCTATCATCTCTACTCTGACTTAACTGCAG
L N R W I T F C Q S I I S T L T *
                               PstI

```

FIG. 15 G

(BABS) -

```

      10      20      30      40      50      60
GGATCCGGTGTCTGACAACAAATTCAACAAGGAACAGCAGAACGCGTTCTACGAGATCTTG
G S G A D N K F N K E Q Q N A F Y E I L
BamHI                               MluI      BglII
                                   XmnI

      70      80      90      100     110     120
CACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAG
H L P N L N E E Q R N G F I Q S L K D E
BspMI+                               HindIII

      130     140     150     160     170     180
CCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCG
P S Q S A N L L A D A K K L N D A Q A P
                                   NheI      FspI

      190     200     210     220     230     240
AAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGGAACAGCAGAACGCG
K S D Q G Q F M A D N K F N K E Q Q N A
                                   MluI
                                   XmnI

      250     260     270     280     290     300
TTCTACGAGATCTTGACCTGCCGAACCTGAACGAAGAGCAGCGTAACGGCTTCATCCAA
F Y E I L H L P N L N E E Q R N G F I Q
BglII      BspMI+                               H

      310     320     330     340     350     360
AGCTTGAAGGATGAGCCCTCTCAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAAC
S L K D E P S Q S A N L L A D A K K L N
indIII                               NheI

      370     380
GATGCGCAGGCACCGAAATAACTGCAG
D A Q A P K *
FspI                               PstI

```

FIG. 15H